University of Alberta

Behaviour of Cold Adapted, Log Phase *Escherichia coli* at Temperatures Near the Minimum for Growth

by

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Abstract

The behaviour of cold adapted, log phase *Escherichia coli* at temperatures near 7°C, the minimum for growth, was investigated because discrepancies were found in the relationship between increases in absorbance values and the number of colony forming units (cfu). At suitable intervals, absorbance readings (A_{600}) were determined, cell length was determined by microscopy and/or flow cytometry, and colonies were enumerated on plate count agar. Cells elongated to form filaments during incubation at constant temperatures >4°C and <15°C. At constant temperatures >7°C, numbers of colony forming units (cfu) and A_{600} increased with time, but at constant temperatures ≤7°C, numbers of cfu decreased while A_{600} increased. When cells incubated at <7°C were incubated at temperatures >7°C, elongated cells lengthened further before dividing into cells of normal size. Cells elongated when cultures were incubated at 4 or 2°C with increases to 10°C for 35 min at 6 h intervals. Elongated cells were able to divide when temperatures were raised from 6°C to >7°C for <45 min. at ≤12 h intervals. Such temperature fluctuations may be experienced by chilled foods during defrosting cycles of retail display cases and may have important implications for understanding of appropriate temperatures for safe storage, assessment of microbiological risks, and for predictive modeling of bacterial growth for chilled foods.

The mechanism for filamentation at low temperatures is not known. Therefore, the proteomic response of *E. coli* at temperatures just below the minimum for growth was examined. A number of proteins that were upregulated are involved in protein folding and degradation, carbohydrate metabolism, electron transport, and the TCA cycle and downregulated proteins were involved in protein synthesis or anaerobic carbon

metabolism and energy generation. Cells appear to invoke the stringent response due to insufficient energy to maintain growth. Polymerization of the cell division protein FtsZ requires guanosine triphosphate (GTP), which may be limiting for cell division because GTP is diverted to guanosine pentophosphate (pppGpp) during the stringent response. DNA replication generally ceases under unfavourable conditions but glucose inhibited division protein B (GidB) was upregulated at 6°C, which may allow DNA replication to continue. These studies provide insight into the physiological response of *E. coli* at temperatures near the minimum for growth.

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Table of Contents

1. Introduction and literature review 1
  1.1 Behaviour of *Escherichia coli* upon exposure to low temperature stress 1
  1.2 Effect of cold stress on macromolecules 3
    1.2.1 Cell membrane 4
    1.2.2 Ribosomes and RNA 4
    1.2.3 Proteins 6
    1.2.4 Compatible Solutes 9
    1.2.5 DNA 12
    1.2.6 CspA family 13
  1.3 Effect of low temperature on cell growth and division 16
    1.3.1 DNA replication 16
    1.3.2 SOS response 18
    1.3.3 DNA segregation 23
    1.3.4 Cell division 24
    1.3.5 Peptidoglycan synthesis 28
  1.4 General stress response 30
    1.4.1 RpoS 30
    1.4.2 Regulation of RpoS 31
  1.5 The stringent response 33
  1.6 Stress and the single cell 36
  1.7 Filamentation as a consequence of sublethal stress 37
  1.8 Conclusions 39
  1.9 Research objectives 41
  1.10 References 43
2. Behaviour of log phase *Escherichia coli* at constant temperatures near the minimum for sustained growth 68
  2.1 Introduction 68
  2.2 Materials and methods 69
    2.2.1 Culture preparation and enumeration of *E. coli* 69

2.2.2 Survival and growth of cold adapted *E. coli* in BHI at temperatures below 7°C and subsequent growth at 12°C 70

2.2.3 Growth of subpopulations of cold adapted *E. coli* cells, enriched for normal or elongated cells formed during incubation at 6°C, at 12°C 71

2.2.4 Survival and growth of *E. coli* in BHI at temperatures between 7°C and 15°C 72

2.2.5 Measurement of cell lengths 73

2.2.6 Flow cytometry 73

2.2.7 Analysis of data 74

2.3 Results 74

2.3.1 Behaviour of cold adapted *E. coli* at temperatures below 7°C 74

2.3.2 Behaviour of *E. coli* at 12°C after incubation at temperatures below 7°C 76

2.3.3 Behaviour of subpopulations of *E. coli* cells at 12°C separated by size after incubation at 6°C 81

2.3.4 Behaviour of cold-adapted *E. coli* at temperatures between 7°C and 10°C 88

2.3.5 Behaviour of *E. coli* between 12°C and 15°C 91

2.3.6 Behaviour of other cold adapted *E. coli* strains at 9°C 94

2.4 Discussion 95

2.4.1 Behaviour of cold adapted, log phase *E. coli* at temperatures below 7°C 95

2.4.2 Behaviour of *E. coli* at 12°C after incubation at temperatures below 7°C 97

2.4.3 Behaviour of cold-adapted *E. coli* at temperatures > 7°C 98

2.5 References 101

3. The behaviour of log phase *Escherichia coli* at temperatures that fluctuate about the minimum for growth 104

3.1 Introduction 104

3.2 Materials and methods 105

3.2.1 Culture conditions 105

3.2.2 Flow cytometry 106

3.2.3 Analysis of data 106

3.3 Results 107

3.4 Discussion 110

3.5 References 112

4. Changes in the proteome of *Escherichia coli* in response to temperatures below the minimum for sustained growth 114

4.1 Introduction 114

4.2 Materials and methods 118

4.2.1 Culture conditions 118

4.2.2 Flow cytometry 119

4.2.3 Preparation of protein extracts 119

4.2.4 Two-dimensional gel electrophoresis 120

4.2.5 Image analysis 121

4.2.6 Protein identification 121

4.3 Results 122

4.3.1 Behaviour of cold adapted *E.coli* cultures at 2 and 6°C 122

4.3.2 Proteomic analysis of cold adapted *E.coli* cultures at 2 and 6°C 122

4.4 Discussion 133

4.4.1 Behaviour of cold adapted, log phase *E. coli* at 2 and 6°C 133

4.4.2 Two-dimensional gel electrophoresis 134

4.4.3 Proteomic analysis of cold adapted, log phase *E. coli* cultures at 2°C 135

4.4.4 Production of cold shock proteins in cold adapted, log phase *E. coli* at 6°C 135

4.4.5 General stress response 137

4.4.6 Induction of the stringent response 139

4.4.7 Quorum sensing signals 140

4.4.8 Protein stability 142

4.4.9 Carbon metabolism 144

4.4.10 Electron transport 146

4.4.11 Iron metabolism 149

4.4.12 DNA replication 150

4.4.13 Conclusions 153

4.5 References 154

5. General discussion and conclusions 166

5.1 References 175

List of Tables

Table 2.1. Behaviour of cold adapted, log phase *E. coli* cultures incubated at temperatures < 7°C for 8 days and after subsequent incubation at 12°C. 75

Table 2.2. Generation times estimated from numbers of cfu recovered and absorbance values from cold-adapted log phase *E. coli* cultures incubated at 8°C, 9°C or 10°C. 90

Table 2.3. Behaviour of wild-type, ATCC 11775, and ATCC 43895 strains of *E. coli* after 3 days of incubation at 9°C. 95

Table 4.1. Behaviour of cold adapted, log phase *E. coli* after 1 day of incubation at 2°C or after 1, 4, or 8 days of incubation at 6°C. 122

Table 4.2. Differential expression and identification of proteins expressed by cold adapted, log phase *E. coli* after incubation at 2 or 6°C. 126

Table 4.3. Identification of proteins, their gene, function and relative expression levels at 6 and 2°C for proteins that display increased expression by cold adapted, log phase *E. coli* after 8 days of incubation at 6°C relative to levels expressed at 15°C........ 128

Table 4.4. Identification of proteins, their gene, function and relative expression levels at 6 and 2°C for proteins that display decreased expression by cold adapted, log phase *E. coli* after 8 days of incubation at 6°C relative to levels expressed at 15°C........ 132

List of Figures

Figure 1.1. Model of the mode of action of CspA during initiation of translation at optimum growth temperature, immediately after cold shock, and after cold adaptation 6

Figure 1.2. Proposed order of events for cell division of *E. coli*. 25

Figure 1.3. Effect of various modulators of transcription, translation, or proteolysis on RpoS activity 32

Figure 1.4. Metabolic pathways of (p)ppGpp synthesis and degradation 35

Figure 2.1. Changes in absorbance values of cold adapted, log phase *E. coli* cultures incubated at 2°C or 6°C for 8 days. 75

Figure 2.2. Numbers of *E. coli* recovered on plate count agar or violet red bile agar from log phase cultures incubated at 2°C or 6°C for 8 days. 76

Figure 2.3. Mean lengths of all or the longest 10% of cells in log phase cultures of *E. coli* incubated at 2°C or 6°C for 8 days. 77

Figure 2.4. Changes in absorbance values of cold adapted, log phase *E. coli* cultures incubated at 12°C or incubated at 12°C after incubation at 2°C for 4 d or at 6°C for 0.5, 4, or 8 d. 77

Figure 2.5. Numbers of *E. coli* recovered on plate count agar from log phase cultures incubated at 12°C or incubated at 12°C after incubation at 2°C for 4 d or at 6°C for 0.5, 4, or 8 d 79

Figure 2.6. Numbers of *E. coli* recovered on plate count agar or violet red bile agar from log phase cultures incubated at 12°C for 48 h after incubation at 6°C for 8 days. ... 80

Figure 2.7. Mean lengths of cells in cold adapted, log phase *E. coli* cultures incubated at 12°C for 48 h after incubation at 2°C for 4 d or after incubation at 6°C for 0.5, 4, or 8 d 82

Figure 2.8. Mean lengths of the longest 10% of cells in cold adapted, log phase *E. coli* cultures incubated at 12°C for 48 h after incubation at 2°C for 4 d or after incubation at 6°C for 0.5, 4 or 8 d. 82

Figure 2.9. Distribution of the lengths of cold adapted, log phase *E. coli* cells incubated at 12°C, or after 26 h or 44 h at 12°C after being incubated at 6°C for 8 days........ 83

Figure 2.10. Morphology of cold adapted, log phase *E. coli* incubated at 12°C, after 8 d at 2°C or after 8 d at 6°C 84

Figure 2.11. Morphology of cold adapted, log phase *E. coli* after 26 h at 12°C after being incubated at 6°C for 8 days 85

Figure 2.12. Percentage of cells giving mean forward angle light scatter measurements less than the reference value of the 90% percentile before incubation at 6°C during subsequent incubation at 12°C in cultures enriched for cells of normal length or elongated cells formed after 6 d of incubation at 6°. 86

Figure 2.13. Mean forward angle light scatter (FALS) measurements and FALS measurements of the longest 10% of cells during incubation at 12°C of cultures enriched for cells of normal length or elongated cells formed after 6 d of incubation at 6°. 87

Figure 2.14. Changes in absorbance values of cold adapted, log phase *E. coli* cultures incubated at 12°C of cultures enriched for cells of normal length or elongated cells formed after 6 d of incubation at 6°. 87

Figure 2.15. Numbers of *E. coli* recovered on plate count agar from log phase cultures incubated at 12°C of cultures enriched for cells of normal length or elongated cells formed after 6 d of incubation at 6°. 88

Figure 2.16. Numbers of *E. coli* recovered on plate count agar from cold adapted, log phase cultures incubated at 7, 8, 9 or 10°C. 89

Figure 2.17. Changes in absorbance values of cold adapted log phase *E. coli* cultures incubated at 7, 8, 9 or 10°C. 89

Figure 2.18. Mean lengths and mean lengths of the longest 10% of cells in cultures of cold adapted, log phase *E. coli* incubated at 7, 8, 9 or 10°C 90

Figure 2.19. Mean forward angle light scatter (FALS) measurements and mean FALS measurements of the longest 10% of cells of cold adapted, log phase *E. coli* cells incubated at 7, 8, 9 or 10°C 91

Figure 2.20. Distribution of forward angle light scatter (FALS) measurements of *E. coli* cells in cold adapted, log phase cultures before and after 5 days of incubation at 7°C. 92

Figure 2.21. Percentage of cold adapted *E. coli* cells in log phase cultures giving forward angle light scatter measurements below the reference value of the 90th percentile before incubation at 7, 8, 9 or 10ºC. 92

Figure 2.22. Mean lengths and mean lengths of the longest 10% of cells in cultures of log phase *E. coli* incubated at 12ºC or 13ºC 93

Figure 2.23. Mean forward angle light scatter (FALS) measurements and FALS measurements of the longest 10% of cells of log phase *E. coli* cells incubated at 12ºC or 13ºC 94

Figure 3.1. Numbers recovered from cold adapted, log phase *E. coli* cultures incubated at constant temperature at 6°C, 4°C or 2°C or at temperatures fluctuating to 10°C at 12 h intervals from 6°C or at 6 h intervals from 4°C or 2°C. 107

Figure 3.2. Changes in absorbance values for cold adapted, log phase *E. coli* cultures incubated at constant temperature at 6°C, 4°C or 2°C or at temperatures fluctuating to 10°C at 12 h intervals from 6°C or at 6 h intervals from 4°C or 2°C. 108

Figure 3.3. Mean forward angle light scatter measurements of the longest 10% of cells in cultures of cold adapted, log phase *E. coli* incubated at constant temperature at 6°C, 4°C or 2°C or at temperatures fluctuating to 10°C at 12 h intervals from 6°C or at 6 h intervals from 4°C or 2°C 109

Figure 3.4. Percentage of cold adapted *E. coli* cells in log phase cultures giving mean forward angle light scatter measurements less than the reference value of the 90th percentile before incubation at constant temperature at 6°C, 4°C or 2°C or at temperatures fluctuating to 10°C at 12 h intervals from 6°C or at 6 h intervals from 4°C or 2°C 110

Figure 4.1. Protein expression profile of cold adapted, log phase *E. coli* after 1 day of incubation at 15°C. 123

Figure 4.2 Protein expression profile of cold adapted, log phase E. coli after 1 day of incubation at 2°C. 123

Figure 4.3. Protein expression profile of cold adapted, log phase *E. coli* after 1 day of incubation at 6°C. 124

Figure 4.4. Protein expression profile of cold adapted, log phase *E. coli* after 4 days of incubation at 6°C. 124

Figure 4.5. Protein expression profile of cold adapted, log phase *E. coli* after 8 days of incubation at 6°C. 125

Figure 4.6. Expression of OsmY at spot 203 after incubation of *E. coli* at 15°C for 1 day followed by incubation at 2°C for 1 day or 6°C for 1, 4 or 8 days. 130

Figure 4.7 Expression of dihydropteridine reductase at spot 672 after incubation of *E. coli* at 15°C for 1 day followed by incubation at 2°C for 1 day or 6°C for 1, 4 or 8 days. 130

Figure 4.8. Expression of GidB at spot 320 after incubation of *E. coli* at 15°C for 1 day followed by incubation at 2°C for 1 day or 6°C for 1, 4 or 8 days. 131

Figure 4.9. Expression of CsdA at spot 1117 after incubation of *E. coli* at 15°C for 1 day followed by incubation at 2°C for 1 day or 6°C for 1, 4 or 8 days. 133

Figure 4.10. Expression of flagellin at spot 893 after incubation of *E. coli* at 15°C for 1 day followed by incubation at 2°C for 1 day or 6°C for 1, 4 or 8 days. 134

List of Abbreviations

A	adenine
A_{600}	absorbance
ackA	acetate kinase
ADP	adenosine diphosphate
AHL	acylated homoserine lactones
AI	autoinducer
AMP	adenosine monophosphate
ampC	ampicillin, β-lactamase
aspA	aspartate ammonia-lyase
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
BHI	brain heart infusion
bolA	bolus, morphogene
bp	base pair
C	cytosine
cAMP	cyclic AMP
cfu	colony forming units
clp	caseinolytic protease
CM	cytoplasmic membrane
CRP	cAMP receptor protein
Crr	phosphocarrier protein for glucose of PTS
csdA	cold-shock DEAD-box protein A
csp	cold shock protein
dac	D-alanine carboxypeptidase
dam	DNA adenine methylase
datA	DnaA titration
DB	down stream box
dcm	DNA cytosine methylation
dif	deletion induced filamentation, site specific recombination

Din	damage inducible, DNA repair enzyme
DNA	deoxyribonucleic acid
dnaA	DNA biosynthesis, initiation protein
DnaB	DNA biosynthesis, chain elongation
DnaJ	DNA chaperone protein
dnaK	DNA chaperone protein (Hsp70)
DnaT	DNA replication primosomal protein I
Dps	DNA binding protein, starvation
ds	double stranded
dsbC	disulphide bond isomerase
DsrA	regulatory RNA
DTT	dithiothreitol
EGR	exponential growth rate
EF	elongation factor
ETC	electron transport chain
FAD	flavin adenine dinucleotide (oxidized form)
FALS	forward angle light scatter
Fis	factor for inversion stimulation, DNA binding protein
fliC	flagellin
frdB	fumarate reductase, iron-sulfur protein subunit
fts	filamentation temperature sensitivity, cell division protein
ftsI	peptidoglycan synthetase (PBP 3)
fur	ferric uptake regulator
G	guanine
GDP	guanosine diphosphate
gid	glucose inhibited division protein, cell division, DNA replication
glmS	glucosamine-6-P-synthetase
gltA	citrate synthase
gpp	guanosine pentaphosphate phosphatase
GroEL	growth of phage, protein chaperone
GroES	growth of phage, protein chaperone

GrpE	growth after prophage induction, heat inducible nucleotide exchange factor
GTP	guanosine triphosphate
gyrA	gyrase
HACCP	hazard analysis critical control point
hfq (HF-I)	host factor for Qβ, RNA binding protein
hmp	dihydropteridine reductase
hns	histone-like protein
HPLC	high pressure liquid chromatography
Hpr	histidine protein
Hsc	heat shock cognate protein
hsdS	host specificity, DNA modification
HslUV	heat shock loci, protease
hsp	heat shock protein
ID	internal diameter
IEF	isoelectric focusing
IHF	integration host factor, transcriptional activator
IMP	inosine monophosphate
IPG	immobilized pH gradient
isc	iron sulphur cluster
iscS	cysteine desulfurase
iSDR	inducible stable DNA replication
kDa	kiloDalton
kVh	kilo volt hour
LB	Luria Broth
LC	liquid chromatography
lexA	lambda excision, global regulator
lon	long form, protease
LuxR	transcriptional activator
luxS	S-ribosylhomocysteinase, autoinducer-2 synthesis

lys	lysine-tRNA synthetase
M	molar
MALDI	matrix-assisted laser desorption/ionization
mdh	malate dehydrogenase
Min	minicell, position of division septum
mM	millimolar
mRNA	messenger RNA
MS	mass spectrometry
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NCBI	National Center for Biotechnology Information
ndk	nucleoside diphosphate kinase
NDP	nucleoside diphosphate
nif	nitrogen fixation
nm	nanometer
NO	nitric oxide
NTP	nucleoside triphosphate
nusA	N (lambda protein) utilization substance, transcription termination and antitermination
oriC	origin of replication
OM	outer membrane
oppA	oligopeptide transport, periplasmic binding protein
osmY	osmotically inducible perimplasmic protein
otsA	osmoregulatory trehalose synthesis, trehalose P synthetase
otsB	osmoregulatory trehalose synthesis, trehalose P phosphatase
OxyR	oxygen, regulatory protein sensor
OxyS	oxygen, regulatory RNA
P	promoter
PBP	penicillin binding protein
PCA	plate count agar
pepB	peptidase B

Pi	inorganic orthophosphate
pflA	pyruvate formate lyase activating enzyme 1
pflB	pyruvate formate lyase I
pHi	internal pH
poxB	pyruvate oxidase
PPi	inorganic pyrophosphate
ppk	polyphosphate kinase
ppx	polyphosphatase
ppGpp	guanosine tetraphosphate
pppGpp	guanosine pentaphosphate
PPIase	peptidyl-prolyl isomerase
ppiB	peptidyl-prolyl cis-trans isomerase B
Pri	primosome
proU	proline, high affinity transport system
psp	phage shock protein
PTS	phosphotransferase carbohydrate transport system
ptsI	phosphotransferase system enzyme I
purB	probable adenylosuccinate lyase
RbfA	ribosome binding factor
RcsB	regulation capsule synthesis
recA	recombination, general recombination and DNA repair
recBCD	recombination, exonuclease
relA	relaxed, ATP:GTP 3'pyrophosphotransferase
rhlB	RNA helicase like
RNA	ribonucleic acid
rph	RNase PH
rpoD	RNA polymerase, housekeeping sigma factor (σ^{70})
rpoH	RNA polymerase, heat shock sigma factor (σ^{32})
rpoN	RNA polymerase, nitrogen fixation sigma factor (σ^{54})
rpoS	RNA polymerase, starvation or stationary phase sigma factor (σ^{38})
rRNA	ribosomal RNA

RssB (SprE, MviA)	regulator of σ^{38}
S	Svedberg unit
sdaA	L-serine deaminase
SdiA	suppress division inhibitor
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIP	standard isotonic Percoll
slyD	sensitive to lysis, PPIase
sucB	2-oxoketoglutarate dehydrogenase
sucC	succinyl-CoA synthetase
spoT	magic spot, guanosine 5'-diphosphate 3'-diphosphate pyrophosphatase; (p)ppGpp synthetase
ss	single stranded
ssb	single-strand DNA-binding protein
SOS	DNA damage rescue response
SoxR	superoxide, regulatory protein
SoxS	superoxide, regulatory protein
sthA	pyridine nucleotide transhydrogenase
sufC	probable ATP-dependent transporter
SuhB	suppressor of heat shock
sulA (sfiA)	suppressor of *lon*, septum formation inhibitor
T	thymine
talB	transaldolase B
TCA	tricarboxylic acid
terC	terminus of DNA replication
TF	trigger factor
TOF	time of flight
tRNA	transfer RNA
t_0	zero time
$t_{(x)}$	time of incubation
U	uracil

umu	UV mutator, error prone repair
uspA	universal stress protein, global regulatory gene
UV	ultraviolet
vol	volume
VRBA	violet red bile agar
w	weight
W	Watt
Xer	*cer*-specific recombination
yjgI	hypothetical oxidoreductase
ZipA	cell division protein
2-D	two dimensional

1. Introduction and Literature Review

1.1 Behaviour of *Escherichia coli* upon exposure to low temperature stress

Bacterial cells undergo cycles of growth and division when environmental conditions are unrestrictive for growth. When a rod shaped cell such as *Escherichia coli* reaches twice its initial length, a septum forms in the middle after the chromosome is replicated, and the cell divides into two daughter cells. Most bacteria are able to adapt to and grow when a variety of changing conditions and stresses affect their environment. However, when environmental conditions become extreme for sustained growth, cells must preserve the integrity of the DNA and maintain an intact cell membrane and proper folding of proteins to maximize their chances for survival (Booth, 2002). To avoid a random arrest of the cell cycle when cells encounter growth limiting conditions, cell mass and components such as DNA and protein no longer increase at the same rate to enable cells to enter the stationary phase in an orderly fashion (Kolter et al., 1993). *E. coli* cells become smaller and develop a spherical instead of a rod shaped morphology during the transition into stationary phase and upon exposure to sudden environmental stresses (Aldea et al., 1989; Santos et al., 1999).

Low temperature is a major factor in the control of the growth of pathogenic and spoilage bacteria in foods. The response of bacteria to low temperature stress depends on the conditions under which they are exposed to such temperatures. While the effect of cold temperature on the behaviour of bacteria that are subjected to a sharp drop in temperature has been well documented, there is a lack of information on the effect of chiller temperatures on cells that are cooled relatively slowly (Jones et al., 1987; Jones and Inouye, 1994). Because mesophilic pathogens such as *Salmonella* and *E. coli* are not expected to grow at temperatures near 7°C (Matches and Liston, 1968; Shaw et al., 1971), limited studies and reviews of adaptation to chiller temperatures have focused on psychrotrophic bacteria such as *Listeria* (Bayles et al., 1996; Liu et al., 2002), *Vibrio* (Araki, 1991; Bryan et al., 1999), *Pseudomonas* (Hebraud et al., 1994; Michel et al., 1997) and *Bacillus* (Graumann et al., 1997; Whyte and Inniss, 1992) because growth of these pathogenic and spoilage bacteria in refrigerated foods is a major concern (Berry and Foegeding, 1997; Gounot, 1991; Wouters et al., 2000). Studies of the cold shock response of exponentially growing *E. coli* cultures have been documented and extensively reviewed (Jones and Inouye, 1994; Panoff et al., 1998; Phadtare et al., 1999; Thieringer et al., 1998). When the temperature is decreased from 37°C to 10°C, growth ceases for about 4 h while cells produce cold shock proteins that allow cells to adapt for subsequent growth at a slower rate at the lower temperature (Jones et al., 1987). Studies of the cold shock response of *E. coli* have concentrated on the initial transient adaptation to cold temperatures but the expression of cold acclimation proteins during continuous growth at low temperatures has not been studied (Phadtare et al., 1999).

A recent study on the behaviour of cold adapted, log phase *E. coli* at temperatures below 7°C indicated that absorbance of broth cultures incubated at nonpermissive temperatures increased at declining rates for several days but corresponding increases in numbers of colony forming units (cfu) as determined by plating were not apparent (Gill et al., 2001). The behaviour of log phase *E. coli* exposed to chiller temperatures appeared to be complex and variable with the chiller temperature, while data for numbers of cfu and absorbance values for the same broth cultures were contradictory (Gill et al., 2001). Despite a single published report of filament formation in *E. coli* cells at temperatures just below the minimum for growth nearly 40 years ago (Shaw, 1968), the existence and implications of this alternate morphological state are just starting to emerge.

Filamentation may be a general stress response to unfavourable environmental conditions (Everis and Betts, 2000; Isom et al., 1995). Filamentation is reported for *E. coli* and *Salmonella* cells at temperatures just below the minimum for sustained growth (Fedio, 1986; Mattick et al., 2003; Phillips et al., 1998; Shaw, 1968) but filamentation has also been reported for gram negative and gram positive bacteria during exposure to high temperature (Rowan and Anderson, 1998), high concentrations of carbon dioxide (McMahon et al., 1998), low and high pH (Everis and Betts, 2001; Isom et al., 1995; Lemay et al., 2000), low water activity (Isom et al., 1995; Jørgensen et al., 1995; Mattick et al., 2000), hydrogen peroxide (Brandi et al., 1989; Isom et al., 1995) and starvation conditions (Giard et al., 2000; Wainwright et al., 1999). Filamentous growth in response to low temperature or low a_w has been attributed to an early block in the cell division genes involved in septation as regularly spaced nucleoids are present, but indentations in the cell wall are not apparent. However, a direct or indirect link to the mechanisms involved in the inhibition of cell division in response to various environmental stresses has not been made (Mattick et al., 2000). Knowledge about the regulation and control of cell morphology during cell growth and division alone is very limited (Young, 2003). Studies of filamentation of *E. coli* cells are predominantly related to induced mutations of various cell division genes (de Boer et al., 1990) or the cell division inhibitor SulA, induced as part of the SOS response when cells are exposed to conditions that damage DNA or interfere with replication (Imlay and Lin, 1987; Walker, 1984; Witkin, 1967). However, there is a lack of knowledge of the mechanism involved in filamentous growth that occurs at temperatures near the minimum for growth.

The objective of this literature review was to examine recent developments in biochemical aspects of cold adaptation and acclimation, and connections to various stress responses and cell division to gain a better understanding of possible mechanisms that may lead to filamentous growth in response to low temperature and other environmental stresses.

1.2 Effect of Cold Stress on Cellular Components

Most bacteria have the ability to grow over a temperature range of about 40°C (Gounot, 1991). Shifts of temperature within the normal growth range generally result in quick, transient adjustments in the level of cell components and enzyme activity but when cells are shifted to temperatures above or below the normal growth range, major changes are necessary for growth. Adaptation and growth of cells at cold temperatures requires that the structural integrity of membranes, proteins and ribosomes is maintained and that the ability to carry out protein synthesis and nutrient uptake is preserved (Berry and Foegeding, 1997). However, a single determinant for adaptation to low temperatures has not been identified (Berry and Foegeding, 1997). The rate of chemical reactions is dependent on temperature, a higher activation energy is required at low temperatures and cells that cannot grow will gradually die as the demand for maintenance energy exceeds the rate of generation (Ingram and Mackey, 1976).

1.2.1 Cell Membrane

The rate of chilling can be critical to survival of bacterial cells exposed to large drops in temperature. The susceptibility to cold shock depends on the unsaturated fatty acid composition of the membrane lipids in relation to crystallization of the membrane lipids (Farrell and Rose, 1968). The integrity of the membrane barrier is maintained by rearrangement of the lipid chains during slow cooling but rapid cooling "fixes" these components in a random, disordered state because membrane lipids undergo a phase transition from liquid crystalline to gel states, possibly creating hydrophobic channels, which result in the loss of the selective permeability of the cellular membrane and membrane leakiness (Leder, 1972; Meynel, 1958; Strange and Dark, 1962). Although *E. coli* can respond to a decrease in temperature by adjusting the membrane lipid composition to incorporate a higher proportion of unsaturated fatty acids to regulate membrane fluidity, the lipid composition does not determine the minimum growth temperature because *E. coli* can be manipulated to grow at 10°C with a lipid composition similar to cells grown at 37°C (Carty et al., 1999; Cronan and Rock, 1987; Shaw and Ingraham, 1965). However, the adjustment in lipid composition in response to lower temperatures is a general phenomenon and may serve some selective advantage (Cronan and Gelmann, 1975).

1.2.2 Ribosomes and RNA

It is well established that a block in initiation of translation is the major factor that limits the growth of mesophiles at low temperatures (Jones and Inouye, 1994). When *E. coli* are shifted from 37°C to 8°C or lower, polysomal runoff and the accumulation of 70S particles and ribosomal subunits occurs, which leaves the cell incapable of initiating protein synthesis (Broeze et al., 1978; Das and Goldstein, 1968; Friedman et al., 1969). When *E. coli* are shifted from 37°C to 10°C, a unique set of proteins is produced during a 4 h lag period while the synthesis of almost all other proteins is inhibited (Jones et al., 1987). This adjustment period is known because the cold shock response. Production of the major cold shock protein, CspA, is increased about 200-fold within the first 1.5 h of the temperature shift from 37°C to 10°C (Jones et al., 1987; Goldstein et al., 1990). The *cspA* mRNA contains a 14-base downstream box (DB) located 12 bp downstream of the initiation codon of *cspA* that is complementary to a region near to the decoding region of 16S rRNA. The DB of *cspA* mRNA plays a crucial role in initiation of mRNA translation during the acclimation phase at low temperature by facilitating the formation of a translation pre-initiation complex through binding to 16S RNA (Mitta et al., 1997). When cold shock occurs, ribosomes become nonfunctional for cellular mRNAs, except mRNAs for cold shock proteins (Mitta et al., 1997). The CspA may act like a RNA chaperone by binding to mRNA with broad specificity to prevent the secondary folding at low temperatures and thus allow the initiation of translation (Fig. 1.1; Jiang et al., 1997). During the lag period, cold-unadapted ribosomes are converted to cold-adapted ribosomes by aquiring cold shock ribosomal factors RbfA (Jones and Inouye, 1996) and CsdA (Jones et al., 1996). These cold-adapted ribosomes are able to translate non-cold shock mRNAs. After the end of the lag period, the production of the cold shock proteins is reduced to a new basal level and the synthesis of non-cold shock proteins is resumed. Even during growth at 15°C, translation does not occur at an optimal rate because the fraction of nontranslating ribosomes in cells is three times higher than during growth at 37°C (Farewell and Neidhardt, 1998).

In contrast, the synthesis of housekeeping proteins is not repressed in psychrotrophs or psychrophiles following an abrupt temperature downshift (Berger et al., 1997). In addition, the relative level of cold shock proteins induced is moderate even after a severe cold shock, and CspA-like proteins, referred to as cold acclimation proteins, are continuously synthesized during prolonged growth at low temperatures, which allows cells to continuously translate mRNA (Araki, 1991; Bayles et al., 1996; Berger et al., 1997; Hebraud et al., 1994; Roberts and Inniss, 1992; Whyte and Innis, 1992). Structural and metabolic proteins of psychotrophs and psychrophiles are required to be functional at low temperatures (Gounot, 1991). Therefore it is anticipated that psychotrophs and psychrophiles respond differently to cold shock than mesophiles because the cold shock response occurs near the minimum temperature for growth of mesophiles but not of psychotrophs and psychrophiles.

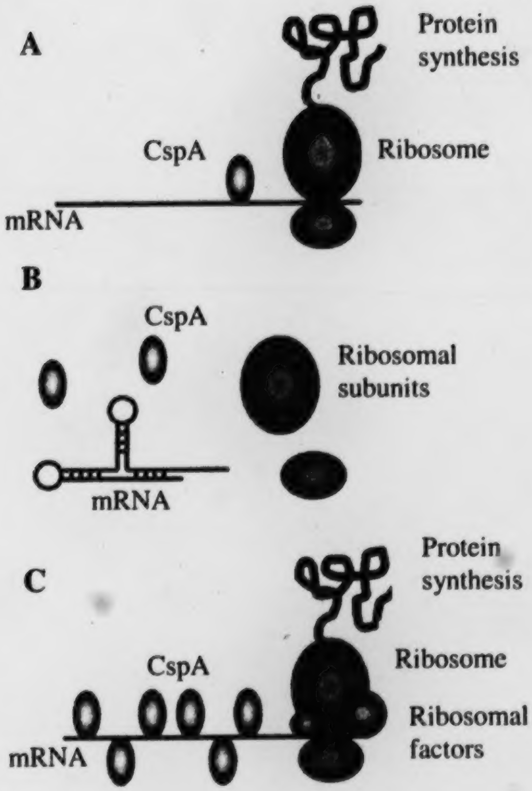
A
Protein synthesis
CspA
Ribosome
mRNA
B
CspA
Ribosomal subunits
mRNA
C
Protein synthesis
Ribosome
CspA
Ribosomal factors
mRNA

Figure 1.1. Model of the mode of action of CspA during initiation of translation at optimum growth temperature (A), immediately after cold shock (B), and after cold adaptation (C). Figure adapted from Abee and Wouters (1999), see text for details.

1.2.3 Proteins

Ribosomal proteins and other structural and regulatory proteins require a precise conformation to carry out their function. The conformational stability of proteins may be changed as hydrophobic bonds become weaker and hydrogen bonds become stronger as temperatures decrease (Jaenicke, 1991). Such alterations in conformation may expose hydrophobic amino acid residues and cause misfolded proteins to aggregate through hydrophobic and hydrogen bonding (Hartl and Hayer-Hartl, 2002). Molecular chaperones protect cells from thermal stress by binding to the exposed hydrophobic amino acid residues of unfolded, misfolded or aggregated proteins and either assist them to refold properly or target them for destruction (Georgopolous and Welch, 1993). Many of the heat shock proteins (hsps) are molecular chaperones and ATP dependent proteases. Molecular chaperones DnaK, GroEL and GroES are required for normal growth at 37°C but expression increases in cells exposed to high temperatures and other environmental stresses that affect protein structure (Georgopolous and Welch, 1993). However, filamentation is observed at 15°C in cells lacking a functional *csdA* gene, which has been linked to lower levels of DnaK and GroEL in the *csdA* mutant strain (Jones et al., 1996). Protein misfolding is accepted as a major problem at high temperatures but it was not considered a major problem at low temperatures until recent studies demonstrated the induction of molecular chaperones trigger factor (TF), Hsc66 and Hsc20 in response to cold temperatures (Kandror and Goldberg, 1997; Lelivelt and Kawula, 1995).

Trigger factor is essential for cell viability at low temperatures and is induced about 2 to 3 h in the lag period that follows cold shock (Kandror and Goldberg, 1997). A higher or lower rate of survival during storage at 4°C for 7 days was observed for strains that produce higher or lower levels of TF, respectively, than the wildtype strain (Kandror and Goldberg, 1997). Trigger factor and DnaK cooperate to promote proper folding of newly synthesized proteins and the lack of either chaperone at 30°C does not have an adverse effect on cell viability but a lack of both chaperones is lethal (Deuerling et al., 1999; Teter et al., 1999). Although TF and DnaK have an overlap in substrate binding specificity by recognizing similar hydrophobic and basic amino acid residues, they are mechanistically distinct (Deuerling et al., 2003). Trigger factor is an ATP independent chaperone associated with the 50S ribosomal subunit and it is the first chaperone that interacts with the nascent polypeptide as it emerges from the ribosome (Blaha et al., 2003; Patzelt, 2002). In contrast, DnaK acts downstream of TF, and it requires ATP and regulatory proteins DnaJ and GrpE to refold misfolded proteins (Bukau and Horwich, 1998). The protective effect of chaperones at low temperatures may be distinct from that at high temperatures because a decrease in viability was observed both when cells that overproduced TF were shifted to 50°C and when cells that overproduced hsps were shifted to 4°C (Kandror and Goldberg, 1997). The findings are not surprising because solubility, aggregation and folding properties of proteins and the strength of hydrogen and hydrophobic bonds are different at high and low temperatures (Jaenicke, 1991).

In addition to chaperone activity, TF also has peptidyl-prolyl-isomerase (PPIase) activity that is essential for protein synthesis and folding to continue at low temperatures. It catalyzes the cis/trans isomerisation of peptide bonds, which is a rate limiting step in the folding of certain polypeptides at low temperatures (Hesterkamp and Bukau, 1996; Stoller et al., 1995; 1996; Teter et al., 1999). The excess TF present in free form in the cytoplasm may play a role in the refolding of cold damaged proteins to functional forms because the rate of synthesis of new proteins is relatively slow (Kandror and Goldberg, 1997; Paztelt et al., 2002).

In addition to promoting proper protein folding, molecular chaperones also play a role in the degradation of abnormal proteins at both low and high temperatures. At low temperatures, TF functions as a co-chaperone because it associates with the ATP dependent chaperone GroEL and enhances its ability to bind to unfolded proteins to promote proper protein folding and degradation (Kandror et al., 1995; 1997). In contrast, during heat shock, GroEL becomes phosphorylated and displays different binding properties for abnormal proteins than when associated with TF, suggesting that heat and cold stress have different effects on the function of GroEL (Kandror et al., 1997).

Relative levels of TF also have an impact on cell morphology (Guthrie and Wickner, 1990); however, the mechanism by which TF affects cell division remains unexplored. Potential roles for chaperone proteins in cell division are discussed in section 1.3.4.

The Hsc66-Hsc20 chaperone system is transiently induced after cold shock, shortly before sustained growth is resumed (Lelivelt and Kawula, 1995; Vickery et al., 1997). The chaperone Hsc66, a 70 kDa hsp homologue with a 62% amino acid similarity to DnaK, is constitutively expressed at levels similar to that of DnaK at normal growth temperatures, but it is not induced on exposure to high temperatures (Lelivelt and Kawula, 1995). The ATPase activity of Hsc66 is regulated by Hsc20, a member of the DnaJ superfamily (Vickery et al., 1997). Unlike DnaK, Hsc66 is not regulated by GrpE (Silberg et al., 1998). However, GrpE is expressed at higher levels at both high and low temperatures, although DnaK is induced after exposure to high but not after exposure to low temperatures (Ang and Georgopoulos, 1989; Zhang and Griffiths, 2003). The chaperones Hsc66 and DnaK carry out different functions because they display different protein binding specificities (Silberg et al., 1998). The Hsc66-Hsc20 chaperone system is involved in the assembly of iron-sulphur cluster proteins but a specific role for the Hsc66-Hsc20 chaperone system has not yet been determined (Hoff et al., 2000). The increased presence of molecular chaperones at low temperatures indicates that protein stability could be a major limiting factor for growth.

1.2.4 Compatible Solutes

Compatible solutes are polar, highly soluble molecules that do not carry a net charge at physiological pH and can be accumulated in the cytoplasm in significant amounts without disturbing the cellular physiology (Bremer and Krämer, 2000). Bacteria can utilize a variety of compatible solutes, such as glycine betaine, ectoine, proline, and trehalose in response to growth phase and growth conditions (Bremer and Krämer, 2000). However, accumulating compatible solutes is energetically expensive (Csonka, 1989).

E. coli and other bacteria possess active transport systems in the cytoplasmic membrane that can accumulate compatible solutes from the external environment when grown in hyperosmotic environments and at low temperatures (Csonka, 1989; Ko et al., 1994; Rajkumari and Gowrishankar, 2001). For *L. monocytogenes*, the transport system for accumulating glycine betaine is activated during growth at low temperatures in broth and on meat, and the presence of glycine betaine enhances its ability to grow at 4°C (Ko et al., 1994; Smith, 1996). In *E. coli*, the ProU transport system accumulates glycine betaine, proline and several related compounds from the external environment in response to increased osmolarity (Csonka, 1989). Transcription of the *proU* operon can proceed from two different promoters: P2 under control of the general housekeeping sigma factor σ^{70}, which is primarily activated under osmotic stress; and P1, which is positively regulated by the alternate stationary phase sigma factor RpoS and negatively regulated by H-NS, which is activated during growth at 10°C (Rajkumare and Gowrishankar, 2001). Expression of *proU* from P1 is influenced by DNA structure at 10°C (Rajkumare and Gowrishankar, 2001).

In addition to active transport, *E. coli* has the ability to accumulate compatible solutes by endogenous synthesis. Trehalose, a nonreducing disaccharide, is synthesized by the cells during the stationary phase and in response to unfavourable environmental conditions such as high and low temperature, osmotic stress, oxidative stress and carbon starvation (Attfield, 1987; Benaroudj et al., 2001; Giaever et al., 1988; Hengge-Aronis et al., 1991; Kandror et al., 2002; Lillie and Pringle, 1980). The enzymes trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) are responsible for cellular trehalose biosynthesis and are encoded by the *otsA/otsB* operon, which is positively regulated by the alternate transcription factor RpoS (Hengge-Aronis et al., 1991; Kaasen et al., 1992).

The steady state production of trehalose in *E. coli* is 3-fold higher at 16°C than at 37°C; however, the increased production at 16°C appears to be of a preventative nature (Kandror et al., 2002). It is not essential for growth or viability at 16°C because similar duration of the lag phase, growth rates, and final cell densities were observed for a trehalose deficient strain carrying a mutation in the *otsA* gene and a wild-type strain (Kandror et al., 2002). Trehalose synthesis does not occur at 4°C but cells with increased trehalose concentrations have higher rates of survival at 4°C (Kandror et al., 2002). Induction of *otsA/B* mRNA at 16°C is dependent on RpoS but independent of the major cold shock protein, CspA (Kandror et al., 2002). Furthermore, *otsA/B* mRNA is more stable at 16°C than at 37°C and, in addition to increased transcription and stability, *otsA/B* mRNA contains the classic DB box that permits the preferential translation of cold shock proteins during the cold adaption phase (Kandror et al., 2002). In contrast to other heat shock and cold shock proteins, OtsA and OtsB are induced at both high and low temperatures.

The accumulation of compatible solutes at low temperatures is beneficial for bacteria, however; the mode of protection is unknown and may differ for each class. While transcription of both *proU* and *otsA/B* are under positive regulation of RpoS at low temperatures, it is not known if both genes are expressed simultaneously or if one is preferentially expressed. Glycine betaine provides cryoprotection to *Listeria* (Ko et al., 1994; Smith, 1996). *E. coli* preferentially accumulates glycine betaine as an osmoprotectant in a rich medium such as Luria broth (LB) and synthesis of trehalose is repressed in the presence of glycine betaine or its precursor choline (Larsen et al., 1987). Even so, cellular levels of trehalose for *E. coli* in LB medium at 16°C had a significant effect on viability when temperatures were reduced to 4°C (Kandror et al., 2002). Moreover, intracellular and extracellular trehalose protects cells from dessication, whereas glycine betaine does not (Welsh and Herbert, 1999).

The mechanism of the protective effect of trehalose has not been elucidated at the molecular level; however, protein denaturation and aggregation are reduced in the presence of trehalose at high temperatures and a similar protective effect may occur at low temperatures (Hottiger et al., 1994; Kandror et al., 2002; Singer and Lindquist, 1998). In addition to induction by high temperatures, hsps are also induced when cells are exposed to compounds that damage cell proteins, such as H_2O_2 and ethanol. Studies with yeast showed that resistance to heat and H_2O_2 were correlated closely with cellular trehalose levels but not with the levels of hsps (Lee and Goldberg, 1998; Benaroudj et al., 2001). When *Saccharomyces cerevisiae* was exposed to the protease inhibitor MG132, hsps and trehalose levels increased and when the inhibitor was removed, hsp production continued to increase but trehalose concentration and the cellular resistance to heat and oxygen radicals decreased rapidly (Lee and Goldberg, 1998; Benaroudj et al., 2001). High levels of trehalose maintain denatured protein in a partially folded state during mild heat stress, which inhibits the reactivation by molecular chaperones until trehalose is degraded (Singer and Lindquist, 1998).

In addition to enhancing protein stability, trehalose has other protective properties, such as protecting proteins, DNA, and membrane lipids from oxidative stress by scavenging for free radicals and enhancing membrane stability, which may or may not be required at low temperatures (Benaroudj et al., 2001, Crowe et al., 1992). Proteins damaged by oxidation contain a higher number of carbonyl groups and strains that were unable to synthesize trehalose contained higher levels of proteins with oxidative damage than cells that produces trehalose (Benaroudj et al., 2001). Trehalose is regarded as a general stress metabolite that may offer protection at low temperatures by multiple mechanisms (Strom and Kaasen, 1993; Van Laere, 1989). Because the accumulation of compatible solutes is a relatively slow process, the cell may first require the activation of a specific stress response system to protect it from the immediate deleterious effects until sufficient amounts of compatible solutes can be accumulated (Welch and Brown, 1996).

1.2.5 DNA

The highly compacted bacterial chromosome is negatively supercoiled in order to retain the energetically favoured double stranded state (Dorman, 1991). Based on the physical chemistry of DNA, underwinding of the strands on the double helix may favour DNA reactions that depend on strand separation such because transcription and replication (Drlica and Zhao, 1997). The bacterial chromosome is subdivided into independently supercoiled domains, suggesting that the location of a gene on the chromosome could influence gene regulation because changes in DNA topology may affect the relative orientation of the -35 and -10 recognition regions for RNA polymerase (Sinden and Pettijohn, 1980). The level of DNA supercoiling is influenced by environmental conditions and increases under anaerobic conditions, increased osmolarity and decreasing growth temperatures (Dorman et al., 1988; Goldstein and Drlica, 1984; Higgins et al., 1988). Expression of *proU* from promoter P1, *recA* and *sulA* are influenced by DNA supercoiling at low temperatures (Dri and Moreau, 1993; Rajkumare and Gowrishankar, 2001; Wang and Syvanen, 1992).

Negative supercoils are made by DNA gyrase, an ATP-dependent topoisomerase II, and the DNA is relaxed by topoisomerase I, using the energy in the supercoiled molecule to drive the reaction. The balance between topoisomerase I and DNA gyrase is thought to maintain negative supercoiling in bacterial cells at levels appropriate for bacterial survival (Menzel and Gellert, 1983). The relative synthesis of the A and B subunit of DNA gyrase is higher after 4 to 5 h at 10°C than at 37°C (Jones et al., 1992b). The A subunit promoter contains 3 recognition sites for the major cold shock protein CspA and is positively regulated by CspA , in contrast, the B subunit is not under CspA control (Jones et al., 1992b). Binding sites for CspA are also found in the promoter region of several other cold shock genes, including *hns* (La Teana et al., 1991), *cspA* (Goldstein et al., 1990), *recA* (Horii et al., 1980), *nusA* (Grandston et al., 1990) and polynucleotide phosphorylase (Regnier et al, 1987). Negative supercoiling by DNA gyrase activates the chromosome for processes that involve strand separation and help replication and transcription complexes move through DNA (Drlica and Zhao, 1997). DNA gyrase also removes knots from DNA and helps to bend and fold DNA (Drlica and Zhao, 1997). The increased synthesis of DNA gyrase may compensate for a decrease in activity at lower temperatures; however, there is a lack of data supporting this suggestion (Golovlev, 2003).

The DNA binding protein H-NS, which has a high affinity for curved DNA (Yamada et al., 1990), is also involved in condensation of the chromosome and can repress the transcription of several genes (Hengge-Aronis, 1996). Similar to *gyrA*, *hns* is positively regulated by CspA (La Teana et al., 1991) and is expressed at higher levels after a temperature shift from 37 to 10°C (La Teana et al., 1991). Growth is impaired in strains carrying mutations in *hns* at 12°C but not 37°C, which suggests that H-NS is required for growth at low temperatures (Dersch et al., 1994).

1.2.6 CspA family

The CspA family of *E. coli*, CspA to CspI, consists of nine homologous proteins that most likely originated from gene duplications and are diversified at the level of gene structure, gene regulation and protein structure and function (Yamanaka et al., 1998). The significance of the gene duplication and their clustered organization on the bacterial chromosome is yet to be uncovered but the various CspA homologues possibly arose from adaptation to different environmental stresses, because *E. coli* can survive wide ranges of temperature, osmolarity and pH (Yamanaka et al., 1998).

Although the presence of CspA has been primarily associated with cold shock, classification of CspA as the major cold shock protein is considered a misnomer because CspA is also produced at high levels at 37°C during early exponential growth (Brandi et al., 1999). In the original studies, cultures were in mid-to-late stages of the exponential growth phase when cold shock was induced (Brandi et al., 1999). The transcription of *cspA* is stimulated by Fis and inhibited by H-NS at 37°C; however, these DNA binding proteins do not appear to play a role during cold shock (Brandi et al., 1999).

Of the nine Csp proteins, only CspA, CspB, CspG and CspI are cold shock inducible, but with different specificities; the maximum levels of induction is between 10°C and 24°C for CspA; at 15°C for CspB and CspG; and between 10°C and 15°C for

CspI (Goldstein et al., 1990; Lee et al., 1994; Nakashima et al., 1996; Wang et al., 1999). Although increased transcription of *cpsG* mRNA is transient at 15°C, steady state expression of CspG remains elevated after 24 h (Nakashima et al., 1996). An unusually long 5' untranslated region in *cspA*, *cspB* and *cspG*, and *csdA* has a positive or negative effect on mRNA stability at 15°C or 37°C, respectively (Mitta et al., 1997). In addition, DB sequences match closely to the 16S RNA binding sequence for cold inducible genes but not for genes that are not cold inducible (Mitta et al., 1997).

The CspA homologues CspC and CspE are produced constitutively at 37°C, whereas CspD is induced during carbon starvation (Bae et al., 1999; Yamanaka and Inouye, 1997). Yet CspE was highly induced during cold shock at 15°C in a strain of *E. coli* that carried a triple deletion for the genes of *cspA*, *cspB* and *cspG*, indicating that CspA homologues, other than CspD, can complement each other to maintain a constant amount of CspA homologues in the cell at low temperatures (Wang et al., 1999; Xia et al., 2001). The CspA homologues negatively regulate each other, CspE acts as a negative regulator for CspA at 37°C and CspE is negatively regulated by CspA, CspB, and CspG at low temperatures (Bae et al., 1999; Xia et al., 2001). When *cspE* was deleted from an *E. coli* strain carrying the triple deletion Δ*cspA* Δ*cspB* Δ*cspG*, cells became filamentous after 72 h at 15°C, and the chromosomes, as many as 60, were normally segregated and evenly spaced (Xia et al., 2001). This indicates a problem with septum formation; however, all CspA homologues, with the exception of CspD, were able to suppress the cell division defect (Xia et al., 2001). The mechanism for cold sensitive cell division is not known. Suggestions that a key cell division protein requires a CspA homologue for efficient expression at low temperatures requires further investigation, although the content of FtsZ, a key cell division protein, was similar in the wild-type and the quadruple deletion strain (Xia et al., 2001). When the filaments were returned to 37°C, the majority divided into cells of normal size; however, a small fraction of cells were irreversibly impaired in cell division and formed even longer filaments (Xia et al., 2001). The CpsA homologues CspC and CspE are positive regulators of stress response proteins RpoS and UspA at 37°C but the mechanism of action is unknown (Phadtare and Inouye, 2001). Slight overexpression of CspC and CspE in exponentially growing cells led to increased stability of *rpoS* mRNA and *uspA* mRNA and increased expression of RpoS and UspA (Phadtare and Inouye, 2001). The increased level of RpoS resulted in increased production of other stress proteins OsmY and Dps (Phadtare and Inouye, 2001). The CpsA homologues CspC and CspE are constitutively expressed at 37°C and are not significantly induced in response to environmental stresses, but as CspA homologues negatively regulate each other and have the ability to complement each other, perhaps other CspA homologues may have a similar regulatory effect on *rpoS* under different environmental conditions (Bae et al., 1999; Phadtare and Inouye, 2001; Xia et al., 2001). However, CspB, CspC, and CspE each have a preferential affinity for different RNA sequences and bind RNA in a more selective manner than CspA (Phadtare and Inouye, 1999).

Expression of *cspD* is not induced by cold shock but it is induced during entry of the cells into the stationary growth phase and during carbon starvation; however, CspD is not essential for growth (Yamanaka and Inouye, 1997; Yamanaka et al., 2001). Unlike many other genes that are induced in the stationary phase, expression of *cspD* is not dependent on RpoS but like *cspA*, it is repressed by H-NS (Yamanaka and Inouye, 1997). Although both CspA and CspD bind to RNA and ssDNA, their biological function and biochemical properties differ. CspA is found as a monomer in solution whereas CspD is found as a dimer (Giangrossi et al., 2001; Newkirk et al., 1994; Yamanaka et al., 2001). Overproduction of CspA does not affect cell growth; however, overproduction of CspD leads to cell elongation and death. The CspA homologue CspD inhibits DNA replication during both initiation and elongation stages and causes tight packing of ssDNA, possibly by binding to the open single stranded regions at the replication fork (Yamanaka et al., 2001).

Low temperatures have a significant effect on the structure and functionality of macromolecules. Mesophilic microorganisms make major changes for adaptation and growth at low temperatures such as modifying the composition of cell membranes and ribosomes and the topology of DNA, inducing molecular chaperones to assist with mRNA translation and protein folding, and inducing systems that accumulate or synthesize compatible solutes, which protect the cell from various other harmful conditions by multiple mechanisms.

1.3 Effect of Low Temperature on Cell Growth and Division

The cycle of cell growth and division has been extensively studied in *E. coli* at or near optimum growth temperatures but very little is known about cell cycle events in cells growing at low temperatures (Atlung and Hansen, 1999). Growth of bacterial cells involves 3 major steps: DNA replication, DNA segregation and cell division. Details of the function and regulation of participating factors are emerging but they have not been completely resolved (Nanninga et al., 2001). Growth appears to be unbalanced at temperatures just below the minimum for sustained growth because many *E. coli* cells become filamentous (Shaw, 1968). When log phase *E. coli* were transferred from 18°C or 10°C to 6°C, synthesis of protein, RNA, DNA and peptidoglycan continued for several days although synthesis of DNA was inhibited slightly before that of other macromolecules; however, DNA synthesis continued in filamentous cells (Shaw, 1968).

1.3.1 DNA replication

The major cell cycle event is the initiation of chromosome replication at the chromosomal origin, *oriC*. Accumulation of a critical concentration of the initiator protein DnaA determines if initiation of chromosome replication occurs (Hansen and Atlung, 1995). The DnaA binds to DnaA boxes within *oriC* and must be bound tightly to ATP for strand separation to occur so that replication can proceed (Bramhill and Kornberg, 1988). The conversion of DnaA-ADP to DnaA-ATP is catalyzed by acidic membrane phospholipids when DnaA is bound to *oriC* while free DnaA is inactivated by phospholipids (Crooke et al., 1992).

The initiator protein DnaA is also a cold shock protein (Atlung and Hansen, 1999). A two-fold higher concentration of DnaA per *oriC* is found in cells grown at 14°C than at 37°C; however, DnaA produced at 14°C has a lower activity than the DnaA produced at 37°C, but the reasons for this are not yet known (Atlung and Hansen, 1999). A higher concentration of DnaA protein appears to bind at the open complex formations when conditions are unfavourable for active complex formation (Langer et al., 1996; Messer et al., 2001). Overproduction of DnaA increases the ratio of unsaturated membrane fatty acids to saturated ones at 28°C; furthermore, acidic unsaturated phospholipids such as cardiolipin and phosphatidyl glycerol decrease the affinity of

DnaA for ATP and *oriC* and inhibit the formation of DnaA-*oriC* complexes containing several DnaA molecules *in vitro* (Makise et al., 2002; Suzuki et al., 1998). The phospholipid composition of the cell membrane plays a role in DnaA binding to *oriC* and could be involved in regulation of DNA replication (Makise et al., 2002).

The stability of the replication fork is dependent on optimal concentrations of DnaA (Morigen et al., 2003). Over 300 DnaA boxes with variable affinity for DnaA have been identified in the *E. coli* chromosome, some of which have been found within the cell division genes *ftsQ* and *ftsA* indicating that DnaA may also play a role in coupling DNA replication with cell division (Masters et al., 1989; Schaper and Messer, 1995). The DnaA boxes containing DnaA titration (*datA*) sites can bind as many as 370 DnaA molecules with high affinity (Kitagawa et al., 1996; Morigen et al., 2003). When the availability of free DnaA becomes moderately limited in cells carrying *datA* plasmids, the rate of replication fork movement increases, although the replication forks may not be properly assembled and are more prone to collapse (Morigen et al., 2003). In contrast, when DnaA becomes severely limited in cells carrying a high copy number of *datA* plasmids, the rate of replication fork movement decreases and the SOS response is induced (Morigen et al., 2003). Replication forks also move more slowly when DnaA is overproduced (Atlung and Hansen, 1993; Morigen et al., 2003; Skarstad et al., 1989). Replication forks may pause, stall or collapse when moving from *oriC* to the terminus and they are inactivated under conditions of normal, aerobic growth without the induction of the SOS response (Cox et al., 2000; Robu et al., 2001). The causes of replication arrest and the reactivation mechanisms are diverse and have yet to be characterized (Maisnier-Patin et al., 2001).

Replication fork reactivation is a general housekeeping function under normal growth conditions (Cox et al., 2000). The reactivation of replication forks may be mediated by DNA recombination through nonmutagenic pathways and does not necessarily require repair of the lesion that caused the breakdown of the replication fork (Cox et al., 2000). Strand breaks can be repaired through recombination but lesions in double stranded DNA may be subsequently repaired or left behind and cause additional problems during the next round of replication (Cox et al., 2000). In addition, replication machinery can be reassembled outside of *oriC* with the involvement of preprimosomal proteins PriA, PriB, PriC and DnaT. These proteins appear to play a similar role to that of DnaA in that they load DnaB into the forming replisome (Sandler and Marians, 2000). PriA-dependent replication restart can occur by different pathways that may be dependent or independent of homologous recombination (Grompone et al., 2003; Jaktaji and Lloyd, 2003). Stalled replication forks are reactivated directly by PriA primosomes without a requirement for recombination in a *gyrB E. coli* mutant (Grompone et al., 2003).

Thus, the initiation of DNA replication and the movement of the replication fork are affected by the levels and activity of DnaA, a protein which is induced by low temperatures. When the replication fork is inactivated, the mechanism of reactivation is dependent on the type of regulatory network that has been induced.

1.3.2 SOS response

The SOS response of *E. coli* is a coordinated increase in the level of expression of about 30 unrelated genes regulated by the LexA repressor in response to conditions that interfere with DNA replication or damage DNA (Walker, 1984; Fernandez de Henestrosa et al., 2000). Most of the proteins involved in the SOS response are involved in DNA replication, DNA repair and the control of cell division, and are produced at basal levels during normal growth (Koch and Woodgate, 1998; Walker, 1996). The timing of expression of SOS regulated genes is related to the affinity of SOS boxes for the LexA repressor (Kuzminov, 1999). The inducing signal of the SOS response appears to be the progress of the replication fork (Sassanfar and Roberts, 1990). Instability of the replication fork produces gaps and breaks and exposes single stranded (ss) replicating DNA (Sassanfar and Roberts, 1990). RecA polymerizes on exposed ssDNA to form a RecA/ssDNA nucleoprotein filament that is activated in the presence of a nucleotide triphosphate (Heuser and Griffith, 1989; Walker, 1996). Interaction of free LexA protein with the RecA nucleoprotein filament stimulates autodigestion of LexA and decreases the pool of LexA, which results in increased expression of SOS regulated genes, including *recA* (Horii et al., 1981; Little, 1984,1993; Koch and Woodgate, 1998). Several genes of the SOS response are not under control of the LexA repressor, some of them are dependent on activated RecA while others are not (Koch and Woodgate, 1998).

The RecA nucleofilament is multifunctional: it is essential in forming the heteroduplex region in the crossover intermediate in homologous recombination; it has regulatory functions for the induction of the SOS response needed for DNA repair; and it plays a role in error-prone mutagenic repair involving replicative bypass of DNA lesions (Koch and Woodgate, 1998). RecA protein is not activated by ssDNA that is normally present on the lagging strand because single stranded DNA binding protein covers all of the ssDNA produced at the replication fork (Walker, 1996). The *recA* gene contains binding sites for CspA and the level of RecA increases three-fold after a temperature shift from 37 to 10°C (Horii et al., 1980; Jones et al., 1987). The induction of RecA during the cold shock response was thought to be linked to induction of RecA by the RecBCD pathway that occurs during the stationary phase because other proteins associated with the SOS response were not induced during the cold shock response (Jones et al., 1987). However, the short term cold shock reponse studies may not reflect what occurs during long term exposure to low temperatures when the SOS response is gradually induced.

Reactivation of replication forks may occur when RecA is involved in recombination dependent DNA replication that occurs during the SOS response (Sassanfar and Roberts, 1990). The SOS response is only induced under conditions of active DNA replication with ssDNA, generated by the action of nucleases involved in DNA repair or when replication forks are unstable (Sassanfar and Roberts, 1990). DNA replication is dependent on optimal function and regulation of replication proteins because imbalances of replication proteins can lead to induction of the SOS response (Ingmer et al., 2001; Morigen et al., 2003). Therefore, exposure to any environmental condition that interferes with DNA synthesis, either directly or indirectly, has the potential to induce the SOS response. A gradual induction of the SOS response allows cells to induce error free repair processes such as nucleotide excision repair and recombinational repair before inducing other repair pathways such as inducible stable DNA replication (iSDR) that may be error prone (Koch and Woodgate, 1998). It has been suggested that strand opening for iSDR initiation occurs by a displacement-loop model, which would produce two newly replicated chromosomes from the template DNA, resulting in three complete chromosomes (Asai and Kogoma, 1994; Kogoma, 1997). iSDR does not involve DnaA and deoxyribonuclease activity of RecBCD but requires DNA helicase activity of RecBCD, the recombination function of RecA and the replication restart primosome PriA (Masai et al., 1994; Kogoma, 1997). iSDR initiates at unique origins of replication, *oriM1* and *oriM2*, located within *oriC* and within *terC*, the region of chromosome termination, respectively (Kogoma, 1997). Stable DNA replication can occur in the absence of concurrent protein synthesis and is normally repressed but it can be induced during the SOS response. iSDR may increase survival of cells under stress conditions; however, the process is prone to replication error (Kogoma, 1997).

Mutagenic repair allows a cell to recover from DNA damage by replicating through unrepaired lesions in the template DNA that cannot be repaired by error free mechanisms. This requires the induction of *umuD* and *umuC* genes and the molecular chaperones GroEL and GroES (Petit et al., 1994; Smith and Walker, 1998). Although various models have been proposed for SOS mutagenesis, the molecular mechanism is poorly understood. The *umuDC* operon is under control of the LexA repressor and UmuD must be activated to UmuD' by RecA mediated cleavage. Unactivated UmuD may act together with UmuC as a DNA damage checkpoint control that delays the resumption of DNA synthesis and cell growth to allow more time for accurate repair systems to remove or process DNA lesions before replication resumes (Opperman et al., 1999). If the damage is too severe, RecA mediated cleavage of UmuD to UmuD' stimulates translesion synthesis to enable the cell to continue its cell cycle to the best of its ability (Opperman et al., 1999). Two UmuD' molecules associate with UmuC to form a UmuD'$_2$C complex. The binding of the processed UmuD'$_2$C complex to the nucleoprotein filament may switch the role of RecA protein from recombinational to a mutagenic one (Sommer et al., 1993). Sequential folding of UmuC by the Hsp70 and Hsp60 complex is required for full activity and may serve in additional regulation of the UmuD'$_2$C complex (Petit et al., 1994). It is not known if TF can act as a substitute for Hsp70 in folding UmuC at low temperatures. UmuC and SOS regulated DinB have error-prone DNA polymerase activity (Reuven et al., 1999; Tang et al., 1999; Wagner et al., 1999). The *umuDC* operon and the cell division inhibitor gene *sulA*, also known as *sfiA*, are the most tightly repressed genes, whose induction increases about 100-fold after a full induction of the SOS response (Mizusawa and Gottesman, 1983; Schnarr et al., 1991; Kuzminov, 1999).

SulA tightly couples chromosome replication and septum formation and its levels are strictly controlled at both the transcriptional and posttranslational levels. Excess production of SulA causes cells to elongate into filaments through a delay in cell division to allow additional time for DNA repair (Mizusawa and Gottesman, 1983; Kuzminov, 1999). SulA inhibits cell division by binding to a key cell division protein, FtsZ (Trusca et al., 1980). Intracellular levels of the LexA repressor and the rate of transcription of *sulA* can vary widely during the growth cycle of wild-type *E. coli* in rich media in the absence of DNA damaging treatments. Increased expression of *lexA* and *sulA* promoters occurs when the internal pH (pHi) of the cell increases, such as when cells approach the stationary phase and when proteolytic cleavage of the LexA repressor occurs when the pHi decreases, such as when stationary phase cells are diluted into fresh medium (Dri and Moreau, 1993, 1994). Dramatically increased expression of the *sulA* promoter in response to phosphate limitation is related directly or indirectly to increased H-NS levels as opposed to decreased LexA levels (Dri and Moreau, 1993, 1994). Furthermore, expression of the *sulA* promoter is higher at 20°C than at 28°C in the presence of excess phosphate, leading to the speculation that *sulA* expression may be influenced by levels of DNA supercoiling, although the promoter region of *sulA* contains 17 nucleotides between the -10 and -35 RNA polymerase recognition sequence and differs from promoters of *recA* and *proU*, which contain 16 nucleotides (Dri and Moreau, 1993; Owen-Hughes et al., 1992; Schnarr et al., 1991; Wang and Syvanen, 1992). However, it is clear that environmental signals other than the SOS response can influence the transcription of *sulA* (Dri and Moreau, 1993, 1994). RpoS does not affect transcription of *sulA* in *E. coli* during phosphate limitation, but it does have a regulatory effect in rich media that may be related to pHi when cells enter the stationary phase (Dri and Moreau, 1993, 1994).

SulA has a half-life of 1.2 min at 37°C due to rapid degradation by the ATP dependent proteases Lon and HslVU, which ensure that cells can resume normal cell division as soon as SulA synthesis is shut off (Charette et al., 1981; Kanemori et al., 1999; Mizusawa and Gottesman, 1983). Interestingly, HslU of the two-component HslVU protease has two antagonistic functions: it possesses chaperone activity that prevents SulA aggregation; and it possesses regulatory activity that mediates HslV proteolysis of SulA (Seong et al., 2000). Lon and HslVU proteases also degrade abnormal proteins and their gene expression is inducible by the heat shock sigma factor RpoH (Bukau, 1993; Gayda et al., 1985; Kanemori et al., 1997). Induction of the heat shock proteins DnaK and GroEL after exposure to UV light and nalidixic acid, conditions that also induce the SOS response, is independent of RecA and LexA but dependent on RpoH (Krueger and Walker, 1984). The relevance of the induction of hsps as a consequence of exposure to conditions that have a detrimental effect on DNA is not clear (Walker, 1996). While increased expression of the *sulA* promoter is observed at 20°C, there is a lack of knowledge on the stability of SulA and on the activity of proteases at lower temperatures although the rate of SulA degradation is much slower at 30°C than at 42°C in a *lon* null mutant strain (Canceill et al, 1990; Dri and Moreau, 1993). Protein folding is a problem at low temperatures, and since Lon and HslVU proteases are induced as part of the heat shock response and require ATP to degrade abnormal proteins, it may be possible that filamentation at low temperatures is a consequence of excessive accumulation of SulA due to decreased degradation even in the absence of DNA damage.

However, cell division is inhibited in cells that constitutively express the *umuDC* operon from a multicopy plasmid at 30°C but cell division is not impaired at 42°C (Opperman et al., 1996). This "cold" sensitivity for growth at 30°C is independent of activated RecA and SulA, but instead is due to problems associated with chromosome separation (Opperman et al., 1996).

Thus, the SOS response can be induced as a result of exposure to any environmental condition that directly or indirectly interferes with the progress of the replication fork during DNA synthesis. The extent of induction of the SOS response is related to the degree of DNA repair that is required to restore normal DNA replication. SulA, which inhibits cell division by binding to FtsZ, is one of the most tightly repressed genes during the SOS response. However expression from the *sulA* promoter is also increased at lower temperatures and by other environmental stresses. Furthermore, RecA is produced at higher basal levels at low temperatures, which suggests that cells may require additional assistance to maintain DNA replication at low temperatures.

1.3.3 DNA segregation

Chromosomal replication is tightly coordinated with cell division. FtsK is a bi-functional protein that links chromosome partitioning through the DNA binding activity of the C-terminal domain with septum formation through the activities of its N-terminal transmembrane domain (Diez et al., 1997; Draper et al., 1998; Wang and Lutkenhaus, 1998). The daughter chromosomes must be separated after termination of replication before cell division can occur. When a chromosome dimer is formed as a result of homologous recombination, the C-terminal domain of FtsK activates resolvase XerCD to introduce an additional crossing-over in the terminus region at the *dif* locus so that the dimer can be resolved into two monomers (Aussel et al., 2002; Barre et al., 2000; Pérals et al., 2001; Recchia et al., 1999; Steiner et al., 1999). In addition, the C-terminal domain has ATPase activity and functions as a DNA motor protein that may provide a rescue mechanism by pumping DNA away from the closing septum (Aussel et al., 2002; Begg et al., 1995).

Induction of *ftsK* during the SOS response is dependent on RecA and LexA while induction during the stationary phase is under positive regulatory control of ppGpp and is indepent of RpoS (Diez et al., 2000; Draper et al., 1998; Navarro et al., 1998; Wang and Lutkenhaus, 1998). The purpose of induction of *ftsK* during the stationary phase is not clear because other cell division genes, such as those of the *ftsQAZ* operon, are not under regulatory control of ppGpp (Diez et al., 2000; Navarro et al., 1998). Induction of *ftsK* during the SOS response may assist cells to resolve dimers created by recombinational repair of stalled or broken replication forks and provides enhanced resistance to DNA damaging conditions (Draper et al., 1998; Wang and Lutkenhaus, 1998). Increased expression of *ftsK* inhibits formation of the FtsZ ring and may represent another form of inhibition of cell division that is independent of SulA during the SOS response (Draper et al., 1998). Because the SOS response becomes repressed during cell recovery, FtsK localizes along with FtsZ at the septum (Wang and Lutkenhaus, 1998). While the C-terminal domain is important for positioning the chromosomal termini, the N-terminal domain of FtsK is associated with the cell membrane and is essential for cell division (Begg et al., 1995; Draper et al., 1998; Li et al., 2003; Liu et al., 1998; Wang and Lutkenhaus, 1998).

1.3.4 Cell division

In gram negative organisms, cell division is a complex process that involves the coordinated constriction of the cytoplasmic membrane, the murein or peptidoglycan layer and the outer membrane, and the formation of a peptidoglycan crosswall at a specific location selected by the MinCDE system (Hu et al., 1999; Margolin, 2000; Yu and Margolin, 1999). While FtsA, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsZ and ZipA have been identified as essential cell division proteins for *E. coli*, the mechanism and regulation of the cell division machinery and septal ingrowth have not been completely elucidated, although several models have been proposed (Höltje, 1998; Margolin, 2000; Nanninga, 1998; Rothfield and Justice, 1997; Rothfield et al., 1999; Young, 2003). Most cell division genes are clustered together with genes for cell wall synthesis at the 2 min region of the bacterial chromosome, which is also known as the division and cell wall region. FtsZ, FtsA, and ZipA are cytoplasmic division proteins (Hale and deBoer, 1997). Other cell division proteins are associated with the cell membrane where FtsK and FtsW span the cytoplasmic membrane several times while FtsI (also identified as PBP3), FtsL, FtsN and FtsQ transverse the cytoplasmic membrane into the periplasm (Begg et al., 1995; Nanninga, 1998; Rothfield et al., 1999).

FtsZ is an abundant cytoplasmic protein that acts at the earliest known step in cell division. It polymerizes into a continuous or discontinuous ring structure at the division site to form some type of cytoskeletal structure that mediates invagination of the septum and disassembles when the septum is completely formed (Fig. 1.2; Addinall et al., 1996; Bi and Lutkenhaus, 1991; Rothfield and Justice, 1997). The signal that triggers assembly of FtsZ is unknown but may be under negative regulation of the MinCD system (Hu et al., 1999; Yu et al., 1999). Conditional mutants of *ftsZ* in *E. coli* produce long filamentous cells that replicate and segregate their chromosomes but do not show division septa or cellular constrictions, while conditional mutants of *ftsA, ftsQ and ftsI* contain broad indentations at regular intervals along the length of the cell (Addinall et al., 1996; Bi and Lutkenhaus, 1992; Taschner et al., 1988). Thus a morphological distinction can be made between smooth filamentous cells that are unable to form a stable FtsZ ring and filamentous cells with indentations that can form the FtsZ ring but experience

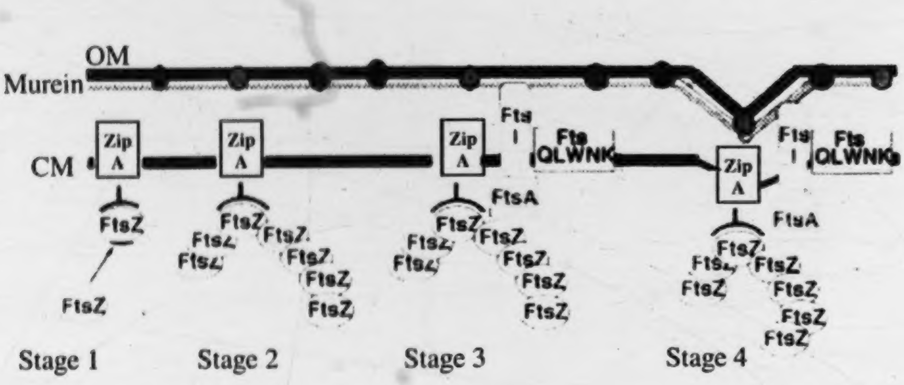

Figure 1.2. Proposed order of events for cell division of *E. coli*. OM, outer membrane; CM, cytoplasmic membrane. Figure adapted from Rothfield and Justice (1997), see text for details.

problems at later stages of cell division (Addinall et al., 1996). Filamentous growth in response to various environmental stresses has been attributed to an early block in the cell division process because regularly spaced nucleoids are present but indentations in the cell wall are not apparent; however, a direct or indirect link to the mechanisms involved in the inhibition of cell division in response to various environmental stresses has not been made. FtsZ ring formation can be prevented by known cell division inhibitors SulA and MinC but may also be influenced by the activity and stability of cell division proteins (Bi and Lutkenhaus, 1993). SulA, induced as part of the SOS response, physically binds to FtsZ, which blocks the GTPase activity and inhibits the polymerization of the FtsZ ring, while MinC prevents the polymerization of FtsZ or perhaps disassembles FtsZ polymers (Hu et al., 1999; Trusca et al., 1980).

After FtsZ localizes at the division site, ZipA and FtsA assemble independent of each other but interact with the C-terminal of FtsZ for localization (Fig 1.2; Addinall and Lutkenhaus, 1996; Liu et al., 1999; Pichoff and Lutkenhaus, 2002). ZipA is a membrane associated protein that may anchor the FtsZ ring to the cytoplasmic membrane (Addinall and Lutkenhaus, 1996; Margolin, 2000). FtsA belongs to a biologically diverse family of ATPases that includes sugar kinases, actin, and Hsp70 proteins but a molecular role for FtsA in cell division has not yet been established (Bork et al., 1992; Margolin, 2000;

Sánchez et al., 1994). Either ZipA or FtsA is capable of supporting formation and stabilization of the FtsZ ring but both are required for recruitment of additional division proteins to the FtsZ ring where FtsK assembles after localization of FtsA and ZipA but before FtsQ and FtsI (Chen and Beckwith, 2001; Pichoff and Lutkenhaus, 2002; Wang and Lutkenhaus, 1998). While *fts*Z, *ftsA*, and *ftsQ* are organized as a gene cluster on the *E. coli* chromosome, major differences in the concentrations of FtsZ, FtsA and FtsQ, estimated at 20,000, 200 and 50 molecules per cell, respectively, may be due to significant differences in protein turnover because differences in efficiencies of transcription and translation alone are not sufficient to account for such differences (Bi and Lutkenhaus, 1991; Mukherjee and Donachie, 1990; Carson et al., 1991; Rothfield et al., 1999). Transcription of the *ftsQAZ* gene cluster can proceed from several promoters, some of which are positively regulated by RpoS, RcsB or SdiA, to allow cells to adjust the division proteins to growth and environmental conditions (Ballasteros et al., 1998; Gervais et al., 1992; Sitnikov et al., 1996; Wang et al., 1991). However, the regulatory effect of SdiA on *ftsQAZ* transcription has been challenged (Michael et al., 2001; Sperandio et al., 2001). The *sdiA* genes of *E. coli* and *Salmonella* encode LuxR homologues that act as a receptor for acyl homoserine lactone signal molecules produced by other species (Michael et al., 2001). Binding of the acyl homoserine lactone signal molecules to SdiA activates transcription of target genes such as *ftsQAZ* (Michael et al., 2001). Transcription of *ftsQAZ* from the "gear box" promoter, which is a promoter sequence that yields a constant amount of gene product per cell and per cell cycle at any growth rate, presumably permits the cell to maintain a constant number of molecules of FtsZ, FtsQ and FtsA per cell when the growth rate falls because cell size is smaller in slowly growing cells (Aldea et al., 1990; Ballasteros et al., 1998; Sitnikov et al., 1996).

The cell must maintain the relative concentrations of cell division proteins within narrow limits to avoid problems during the division process (Dai and Lutkenhaus, 1991; 1992; Dewar et al., 1992; Draper et al., 1998; Hale and de Boer, 1997). Overproduction of ZipA inhibits cell division, which can be suppressed by simultaneous overproduction of FtsZ (Hale and de Boer, 1997). In addition, an increase and a decrease in the ratio of FtsZ to FtsA results in complete inhibition of cell division (Dai and Lutkenhaus, 1992;

Dewar et al., 1992). Furthermore, overproduction of FtsK leads to inhibition of FtsZ ring formation (Draper et al., 1998).

Proper protein conformation of cell division proteins is also essential for cell division. Molecular chaperones play an important role in cell growth, which presumably includes division, by promoting proper protein conformation (section 1.2.3). Filamentation was observed at 30°C in cells lacking a functional DnaK gene but cell division was restored when FtsZ was overexpressed, although slow growth and poor viability indicated that other cellular functions were still impaired due to the lack of DnaK (Bukau and Walker, 1989). TF is a molecular chaperone with PPIase activity that is essential for viability at low temperatures (Kandror and Goldberg, 1997). The relative concentration of TF to FtsZ is crucial for cell division because overproduction or underproduction of TF results in filamentation in cells of strains created to control TF expression (Guthrie and Wickner, 1990). Filamentation induced as a result of overproduction of TF can be suppressed by simultaneous overproduction of FtsZ (Guthrie and Wickner, 1990). SlyD belongs to the same family of PPIases as TF and overexpression of *slyD* also interferes with cell division and leads to filamentation (Roof et al., 1997). SlyD PPIase activity is inhibited by metal ions such as Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+}; however, the physiological role for SlyD is not known (Mitterauer et al., 1999; Roof and Young, 1995). Little is known about the cellular function of PPIases although the *cis-trans* isomerization of peptidyl-prolyl bonds is thought to be a rate limiting step in protein folding. Although direct evidence is lacking, speculation of the involvement of chaperones in cell division is further supported by evidence that the eukaryotic cytoplasmic TCP-1 chaperonin plays a role in the folding and assembly of tubulin microtubules and FtsZ, which has been identified as a homologue of tubulin, forms similar protofilaments *in vitro* to microtubule protofilaments (Erickson et al., 1996; Yaffe et al., 1992).

The process and regulation of cell division is highly complex. In order for cell division to occur, the cell division proteins must be produced in proper relative concentrations and they must be present in a functional conformation. Filamentation is often observed when cell division proteins are unstable or they are present in ratios that are not favourable for cell division.

1.3.5 Peptidoglycan synthesis

In addition to its structural role, FtsZ may have a regulatory function because polymerization of FtsZ appears to act as a signal that directs the localization and synthesis of septal peptidoglycan; therefore, accurate localization of the FtsZ ring may be important in maintaining proper cell shape (Rothfield, 2003; Young, 2003). While single strands of new peptidoglycan are inserted diffusely between old strands during cell elongation, multiple peptidoglycan strands are inserted side by side in a sharply localized growth zone during formation of the septum (Taschner et al., 1988; de Pedro et al., 1997). Peptidoglycan synthesis requires the action of various enzymes, including those with transglycosylase, transpeptidase and endopeptidase activity (Höltje, 1998). Both PBP2 and PBP3 (FtsI) have transpeptidase activity but PBP2, which acts during elongation, requires pentapeptide precursors as acceptors while PBP3, which acts during septum formation, uses both pentapeptide and tripeptide precursors (Nanninga, 1998; de Pedro et al., 2001; Pissabaro et al., 1986). Septal peptidoglycan synthesis is a prerequisite for FtsZ ring constriction (Chen and Beckwith, 2001). Peptidoglycan synthesized at the septum is inert, so that new strands can not be inserted once septation is complete, but the chemical composition seems to be similar to that of the cell wall (de Pedro et al., 1997; Young, 2003).

Incomplete septum formation, false division points and occasional branching in filamentous cells were observed in electron micrographs of log phase *E. coli* after several days at temperatures just below the minimum for growth (Shaw, 1968). Recent investigations suggest that branch formation is rare and is caused by abnormalities in cell elongation rather than disturbances in the cell division process (Gullbrand et al., 1999; de Pedro et al., 2003). Bulges, kinks, and branches contained patches of inert peptidoglycan (de Pedro et al., 2003). Because the earliest observable step in septal development appears to be the synthesis of an inert hoop of peptidoglycan that is dependent on FtsZ, it is speculated that if FtsZ polymerizes at atypical sites or if FtsZ polymerization is aborted, as may well be the case at low temperatures, the patches of inert peptidoglycan may develop into branches or other abnormal cell wall structures (de Pedro et al., 1997; Young, 2003).

Branch formation was enhanced in the presence of aztreonam, a cell division inhibitor that specifically inhibits PBP3, in a strain of *E. coli* that carried a mutation in *dacA*, the gene coding for PBP5 (Nelson and Young, 2000; de Pedro et al., 2003). PBP5 and PBP6 are major peptidoglycan D,D-carboxypeptidases that regulate the number of pentapeptide side chains available for crosslinking during peptidoglycan synthesis by removing the terminal D-alanine (Nelson and Young, 2000). The activity of D,D carboxypeptidase may be a limiting factor in providing the cell with sufficient amounts of tetra-tetra cross links required for PBP3 transpeptidase activity (Begg et al., 1990; Höltje, 1998). Cells carrying the *dacA* mutation divided normally despite the presence of distorted bands of new peptidoglycan at the division site (de Pedro et al., 2003). Furthermore, a division defect in a *ftsK* mutant is suppressed by deletion of the *dacA* gene (Begg et al., 1995). While PBP5 and PBP6 are not essential for cell division, they appear to play an important role in maintaining normal cell morphology because overproduction of D,D carboxypeptidase causes cells to become spherical (Markiewicz et al., 1982). However, it remains to be determined if branching at low temperatures is a consequence of decreased D,D carboxypeptidase activity or some other disturbance in the synthesis of the cell wall or perhaps an indirect effect of the inability to form a stable FtsZ ring.

The *bolA* gene is involved in switching between cell elongation and septation systems, causing a round morphology when cells are exposed to environmental stress (Aldea et al., 1988, 1989). The *bolA* gene product is a regulator of *dacA*, *dacC*, and *ampC*, that encode PBP5, PBP6, and AmpC, respectively (Santos et al., 2002). The round morphology associated with overexpression of *bolA* is dependent on active FtsZ (Aldea et al., 1988). Increased production of BolA protein induces production of higher levels of PBP5 during the early exponential and late stationary growth phase and considerably higher levels of PBP6 during entry into the stationary phase (Santos et al., 2002). Transcription of *bolA* is constitutive from promoter P2 and inducible from the main promoter P1 (Aldea et al., 1989; Lange and Hengge-Aronis, 1991). Growth rate and growth phase dependent expression of *bolA* from promoter P1 is dependent on RpoS while expression from P1 when cells are exposed to environmental stress is dependent or independent of RpoS (Aldea et al., 1989; Lange and Hengge-Aronis, 1991; Santos et al., 1999). The stress induced overexpression of *bolA* may override the growth rate or growth phase dependent regulation to increase cellular resistance and protect cells from environmental stress by reducing the exposed surface area provided that cells are able to form an FtsZ ring (Aldea et al., 1988; Santos et al., 1999).

Thus, FtsZ polymerization may act as a signal for synthesis of the septal peptidoglycan, which in turn is required for constriction of the FtsZ ring. Increased production of BolA, which occurs when cells are exposed to environmental stress or in the stationary phase of growth, increases production of septal peptidoglycan that results in a round morphology in the presence of active FtsZ. However, abnormal cell morphology is observed when the polymerization of FtsZ is impaired or inhibited or when peptidoglycan synthesis is impaired.

1.4 General Stress Response

In addition to the induction of specific stress responses that help cells to cope with that specific stress, *E. coli* is also able to induce a general stress response that provides cross protection against multiple stresses. The general stress response can serve both as an immediate and as a long term strategy of adaptation to various stresses that involve dramatic changes in cellular physiology and morphology, structural changes in the cell envelope, altered membrane composition, and differences in DNA supercoiling and compactness to prevent cellular damage rather than repairing it (Hengge-Aronis, 1996b). Many aspects specific to stationary phase, starvation, osmotic shock, acid shock, heat shock, and cold shock have been attributed to the starvation or stationary phase sigma factor, σ^S (RpoS), product of the *rpoS* gene (Loewen et al., 1998; Tanaka et al., 1993). Apart from its role in stationary phase gene regulation, RpoS is recognized as a global regulator of gene expression under various stress conditions (Hengge-Aronis, 1996a).

1.4.1 RpoS

Many RpoS dependent genes encode proteins that have a role in general stress resistance because inactivation of RpoS increases sensitivity to heat shock, oxidative stress, starvation, acid, ethanol, and near UV light (Farewell et al., 1998; Hengge-Aronis, 1996a). In *E. coli*, RpoS directs the synthesis of more than 50 proteins during the transition from exponential to stationary phase (Loewen et al., 1998). The physiological consequences of the general stress response are not necessarily identical under different stress conditions because the expression of some RpoS dependent genes may also be controlled by various co-regulators that may be present or absent under different stress conditions (Hengge-Aronis, 2000). Moreover, some stress conditions induce RpoS independent responses and the general stress response (Hengge-Aronis, 2000).

The general stress response is usually accompanied by a reduced growth rate or entry into the stationary phase. The switch from gene expression during the exponential phase to expression during the stationary phase requires a change of sigma factors in the holoenzyme RNA polymerase from RpoD to RpoS. RpoS is a highly unstable protein and it is almost undetectable in log phase cultures under non-stress conditions but under stress conditions it increases to 30 to 50% of the level of RpoD (Jishage and Ishihama, 1995; Loewen et al., 1998). The alarmone (p)ppGpp, which accumulates during the stringent response, can act as a positive effector of gene expression and is essential for the induction during the stationary phase and starvation of a large number of genes that are dependent on RpoD (Xiao et al., 1991; Nyström, 1994). Furthermore, RpoS dependent promoters require (p)ppGpp for induction in the stationary phase (Kvint et al., 2000). Stringent conditions may allow alternative sigma factors such as RpoS, RpoH and RpoN to compete more successfully for RNA polymerase, but how that occurs is not clear (Kvint et al., 2000; Jishage et al., 2002).

1.4.2 Regulation of RpoS

The cellular levels of RpoS are controlled by a series of complex mechanisms that affect transcription, translation and post-translational stability (Fig. 1.3), with different stress conditions differentially affecting the various levels of control (Hengge-Aronis, 2000). A gradual deterioration of environmental conditions appears to stimulate *rpoS* transcription or translation while potentially lethal conditions seem to affect RpoS proteolysis (Hengge-Aronis, 2000). The transcription of *rpoS* is inversely correlated with growth rate and positively correlated with (p)ppGpp levels (Lange and Hengge-Aronis, 1994; Lange et al., 1995). While levels of (p)ppGpp increase when cells encounter limiting amounts of available energy, carbon source, or amino acids, (p)ppGpp levels decrease during a temperature downshift (Cashel et al., 1996; Jones et al., 1992a).

Low temperature, high osmolarity and the transition to stationary phase stimulate *rpoS* mRNA translation (Fig. 1.3; Lange and Hengge-Aronis, 1994; Muffler et al., 1996c; Sledjeski et al., 1996). The ribosome-binding site and initiation codon of *rpoS* are located in regions of secondary stem and loop structure, making them inaccessible for ribosome binding (Lange and Hengge-Aronis, 1994). RNA binding protein Hfq (HF-I), encoded by *hfq* and induced by environmental stress, can disrupt the secondary structure of *rpoS* mRNA, thereby increasing the frequency of translational initiation (Brown and Elliot, 1996; Muffler et al., 1996b; Schuppli et al., 1997; Tsui et al., 1994).

Another factor involved in *rpoS* mRNA translation is H-NS, which has an antagonistic effect on Hfq-mediated stimulation of *rpoS* translation and has been

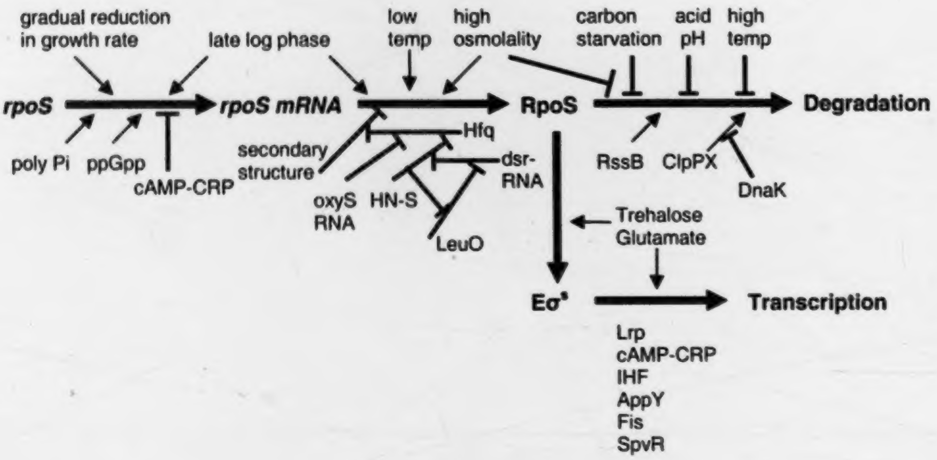
gradual reduction in growth rate
late log phase
low temp
high osmolality
carbon starvation
acid pH
high temp
rpoS
rpoS mRNA
RpoS
Degradation
poly Pi
ppGpp
cAMP-CRP
secondary structure
Hfq
dsr-RNA
oxyS RNA
HN-S
LeuO
RssB
ClpPX
DnaK
Trehalose
Glutamate
Eσs
Transcription
Lrp
cAMP-CRP
IHF
AppY
Fis
SpvR

Figure 1.3. Effect of various modulators of transcription, translation, or proteolysis on RpoS activity. Positive and negative modulators are indicated by arrows and bars, respectively. E = core RNA polymerase. The modulators that are listed below the arrow pointing from $E\sigma^s$ influence $E\sigma^s$ transcription of certain genes [This figure is adapted from Loewen et al. (1998) and Hengge-Aronis (2000)].

associated with a decrease in the levels of RpoS in exponential growth phase (Barth et al., 1995; Nogueira and Springer, 2000; Yamashino et al., 1995). The cold shock protein CspA is a transcriptional activator of *hns* and growth is impaired at 12°C in cells carrying a mutation in *hns* (Dersch et al., 1994; La Teanna et al., 1991). DsrA RNA, a small regulatory RNA, can interfere with the transcriptional silencing action of H-NS and promote translation of RpoS (Majdalani et al., 1998; Sledjeski and Gottesman, 1995; Sledjeski et al., 1996). At 20°C, the accumulation of RpoS in log phase cultures is associated with increased DsrA levels although RpoS or DsrA are not essential for growth under those conditions (Sledjeski et al., 1996). While DsrA interferes with H-NS synthesis by increasing *hns* mRNA turnover through RNA-RNA interactions, binding of DsrA to *rpoS* mRNA increases its stability and enhances *rpoS* translation (Lease and Belfort, 2000a; 2000b). In contrast, OxyS, a small regulatory RNA that integrates the oxidative stress response with other cellular stress responses, inhibits *rpoS* translation by interfering with the activity of Hfq (Zhang et al., 1998).

RpoS is rapidly degraded by proteolysis in unstressed exponentially growing cells but the stability of RpoS increases when cell are suddenly exposed to low pH, high temperatures, high osmolarity, or carbon starvation (Bearson et al., 1996; Lange and Hengge-Aronis, 1994; Muffler et al., 1996c; 1997). The response regulator RssB (also identified as SprE or MviA) binds to a proteolytic-sensitive (turnover) element that is present in RpoS but absent in RpoD, presenting RpoS to ClpXP protease for degradation (Bearson et al., 1996; Becker et al., 1999; Muffler et al., 1996a; Pratt and Silhavy, 1996). The affinity of RssB for RpoS is regulated by phosphorylation of RssB in response to environmental signals (Becker et al., 1999). DnaK is involved in protecting RpoS from degradation by ClpXP but the mechanism of action is not well understood (Muffler et al., 1996b). Regulation of RpoS appears to be very complex and differs for various stresses but a model of the interaction between global regulators of RpoS is starting to emerge (Lease and Belfort, 2000a; Nogueira and Springer, 2000).

1.5 The stringent response

When *E. coli* experiences conditions of limiting amounts of available energy, carbon source, or amino acids, a stringent response is invoked (Cashel et al., 1996). This response involves the accumulation of the alarmone (p)ppGpp, which triggers changes that allow for long term survival and general resistance to various other environmental stresses, including unfavourable temperatures, pH, osmolarity and exposure to reactive oxygen and nitrogen species (Moat et al., 2002). The initial response to carbon-energy source limitation is the appearance or increased expression of high affinity nutrient utilization systems, with scavenging of carbon and energy sources and increased metabolic efficiency. These metabolic changes are mediated by the accumulation of (p)ppGpp and regulated by RpoS and additional global regulators (Moat et al., 2002). Other physiological changes that occur during the stringent response include the degradation of cellular RNA, proteins, and fatty acids; alteration of the lipid composition of the cell membrane; and the condensation of chromosomal DNA, which protects it from damage (Cashel et al., 1996).

Steady state levels of (p)ppGpp are synthesized and maintained in the cytoplasm by phosphorylation of GDP and GTP, with ATP as an energy donor, catalyzed mainly by RelA, which is associated with a small number of ribosomes, and some by SpoT, while hydrolysis is catalyzed by SpoT, which is a cytoplasmic protein (Fig. 1.4). Regulation of (p)ppGpp accumulation in response to energy source limitation is mediated by inhibition of SpoT, which degrades (p)ppGpp to GTP or GDP by an unknown mechanism (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974). Double deletion mutants of *relA* and *spoT* do not synthesize detectable amounts of (p)ppGpp and have similar growth rates as the wild-type at temperatures below 30°C. However, such cultures contain a mixture of elongated cells and short cells, although it is not clear if the effect is solely due to lack of (p)ppGpp (Jones et al., 1992a; Xiao et al., 1991; Cashel et al., 1996).

Degradation of (p)ppGpp is impaired not only under stringent conditions but also by osmotic shock (Harshman and Yamazaki, 1971), heat shock (Gallant et al., 1977), inhibition of fatty acid synthesis (Seyfzadeh et al., 1993), uncouplers of oxidative phosphorylation (Tetu et al., 1980), and long-chain alcohols (Mitchel and Lucas-Lenard, 1980). Conversion of GDP to GTP, catalyzed by nucleoside diphosphate kinase (Ndk), regenerates GTP for pppGpp synthesis (Cashel et al., 1996).

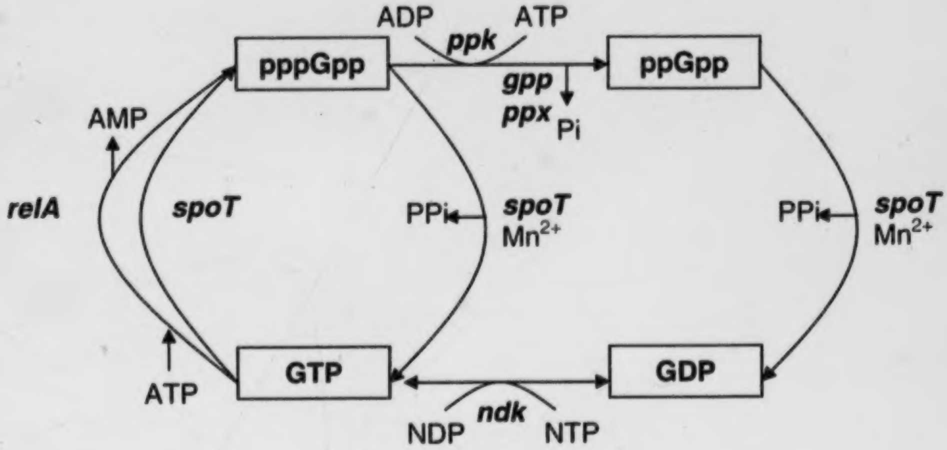

Figure 1.4. Metabolic pathways of (p)ppGpp synthesis and degradation. [This figure is adapted from Cashel et al. (1996)].

As the stringent response progresses past the initial stages, severe inhibition of pppGpp synthesis probably occurs. However, the cause of this inhibition is not known (Cashel et al., 1996). In addition to decreased rates of rRNA accumulation during the stringent reponse, biosynthesis of carbohydrates, lipids, nucleotides, peptidoglycan, and glycolytic intermediates are reduced and the transport of macromolecular precursors into the cell is also shut down (Cashel et al., 1996). Such metabolic changes enhance cellular viability and allow a rapid recovery and initiation of growth when conditions improve (Moat et al., 2002). The degradation of (p)ppGpp is poorly understood but requires the presence of Mn^{2+} (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974; Cashel et al., 1996). Enzymes capable of degrading pppGpp include guanosine pentaphosphate phosphatase (Gpp), polyphosphate kinase (Ppk), and polyphosphatase (Ppx; Wanner, 1996). However, only Ppk is able to transfer the terminal phosphoryl group to ADP to generate ATP. Energy is not conserved when pppGpp is hydrolyzed by Gpp or PPx, (Wanner, 1996). Gpp converts pppGpp to ppGpp and p_i (Belitskii and Shakulov, 1982). Overproduction of Gpp results in abnormally low or nearly undetectable levels of pppGpp during the stringent response (Cashel et al., 1996). The *gpp* gene is downstream of *rhlB* in a dicistronic transcription unit (Kalman et al., 1991). RhlB belongs to the same

DEAD box family of ATP dependent RNA helicases as CsdA, which is dramatically induced at low temperatures (Jones et al., 1996; Toone et al., 1991). However, RhlB has not been identified as a cold inducible protein.

The stringent response can be invoked when cellular demand for energy is larger than the amount that is generated. It has been suggested that (p)ppGpp may help the cell with proper timing of *rpoS* expression for normal stationary phase development (Cashel et al., 1996).

1.6 Stress and the Single Cell

It is difficult to define what constitutes stress from normal conditions because the physiological state of bacteria seems to influence the ability of cells to grow or survive under various environmental conditions. A broad definition suggested by Booth (2002) proposes that "stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potential". The cellular response depends on the severity of the stress. Under slight stress, growth continues at the same rate and cells fully adapt to the new conditions; under severe stress, the growth rate is reduced but cells adapt and tolerate the conditions while under extreme stress, growth ceases and cells switch to a survival mode (Storz and Hengge-Aronis, 2000).

When cultures grow exponentially, the majority of cells double their mass and divide at the same rate, thus supporting the assumption of homogeneity of cells in the culture (Booth, 2002; Davey and Kell, 1996; McKellar, 1997). However, the physiological variation between individual cells becomes apparent when cells become stressed because they do not behave in a similar manner (Booth, 2002; Stephens et al., 1997). Consequently, the behaviour of cells is generally unpredictable when very low numbers are used in an inoculum while large numbers of cells in an inoculum give predictable results (Bridson and Gould, 2000; Stephens et al., 1997). Typical growth curves describe the fate of a bacterial population as opposed to that of individual cells; however, knowledge of the behaviour of the few surviving cells is essential when dealing with bacteria that have a low infectious dose. Although it is impossible to predict which cell will survive, it has been proposed that the surviving cells may have a different balance of proteins than those cells that do not survive (Booth, 2002). Proteins that are present in low abundance, such as regulatory, repair, and stress correction proteins, may be partitioned between cells in unequal proportions during cell division, which could lead to unequal chance of survival during exposure to stress (Booth, 2002). In addition or alternatively, this unequal balance of proteins could be the result of some cells carrying a duplication of a segment of the chromosome, which would increase the mRNA resulting in enhanced expression of proteins advantageous to survival (Booth, 2002). Furthermore, cells may be affected differently at various stages of the growth and division cycle (Kenward and Brown, 1978). It is not known if cell elongation under stress conditions is a deliberate protective mechanism that is advantageous to the cell or if the cell has lost its ability to divide as a result of the stress imposed.

1.7 Filamentation as a consequence of sublethal stress

When cultures of *Salmonella enterica* serovar Enteritidis PT4 and *Salmonella enterica* serovar Typhimurium DT104 that were exposed to low a_w in broth or surfaces were stained with the Live/Dead *Bac*Light viability kit, which differentiates between intact and compromised membranes, a fraction of filamentous cells had intact membranes and were considered viable while nonfilamentous cells were not (Kusumaningrum et al., 2002; Mattick et al., 2000). Filamentation occurred at a_w levels ranging from 0.93 to 0.98 at both 21°C and 37°C in both log phase and stationary phase cells and was independent of *rpoS* expression; however, *rpoS* mutant strains had lower rates of survival than strains containing the *rpoS* gene (Mattick et al., 2000). Filaments as long as 200 µm formed in the presence of NaCl, glycerol or sucrose and divided into cells of normal size after a transfer to more favourable a_w levels (Mattick et al., 2000). Filament formation in various *Salmonella enterica* serovars, including *S.* Enteritidis PT4 and *S.* Typhimurium DT104, occurred at low temperatures in tryptic soy broth, chicken meat extract, chicken meat surfaces and skim milk, irrespective of whether strains carried the *rpoS* gene; however, more extensive filamentation was observed in strains carrying the *rpoS* gene (Fedio, 1986; Mattick et al., 2003; Phillips et al., 1998). In fact, when more than 30 strains that included 20 different serovars of *Salmonella* were examined, all strains were able to form filaments at refrigeration temperatures, indicating that filamentation of mesophilic pathogens at low temperatures may be a general phenomenon (Mattick et al., 2003). Pathogenicity of *S.* Enteritidis PT4 has been linked to presence of the *rpoS* gene (Humphrey et al., 1996; 1998). Log phase cells of *S.* Enteritidis PT4 incubated overnight at 4°C were more heat and acid sensitive but were as pathogenic as stationary phase cells held at 37°C (Humphrey et al., 1998). However, the pathogenicity of filamentous cells formed after several days at low temperatures is not known. Strains of *Aeromonas hydrophila* form chains or filaments in the presence of 100% CO_2 at temperatures ranging from 5 to 28°C, but not when cells are grown aerobically (McMahon et al., 1998). The ability of *A. hydrophila* to produce haemolysin was lost in filamentous cells during exposure to CO_2 but it was regained during subsequent aerobic incubation (McMahon et al., 1998). Unfortunately, elongation in that study was examined when cells had entered the stationary phase, which could result in a minimum elongation effect because cells tend to round off if they retained that ability (Aldea et al., 1989; Santos et al., 1999). However, a fraction of stationary phase *E. coli* cells suspended in water to simulate starvation developed extreme pleomorphism and formed large abnormal filaments with bulbous ends after 5 days of incubation on silicon wafers, plastic, glass or titanium surfaces at 37°C (Wainwright et al., 1999). It would be beneficial to gain information on the subsequent behaviour of the unusual filaments when moved to favourable conditions, because this could have relevance in a food processing environment.

Cell elongation without indentations but not chain formation has been reported for *Salmonella* and *E. coli* at low temperature and low a_w (Mattick et al., 2000; 2003; Shaw, 1968; Wainwright et al, 1999). It is likely that the basis for elongation is different during starvation conditions or exposure to CO_2. Similarly, the morphology of filamentous cells in published images of *Listeria monocytogenes* varies according to the stress applied although it is not known if differences are due to strain variation. Long smooth filaments form in the presence of high NaCl levels and long chains of cells form after exposure to high temperatures (Isom et al., 1995; Jørgensen et al., 1995; Rowan and Anderson, 1998). Rough cell variants (R-forms) of *L. monocytogenes* serotype 4b consist of long chains of cells containing unseparated septa between individual cells that suggests a deficiency in murein hydrolase. These cells are more heat tolerant than the smooth variants (S-forms), consisting of single or paired cells (Rowan and Anderson, 1998). The relative pathogenicity of the R-forms is uncertain because they adhere to and invade eptithelial human colon carcinoma cells but lost the ability to invade phagocytic 3T6 mouse fibroblast cells (Bubert et al., 1992; Kuhn and Goebel, 1989). Cells of *Listeria* subjected to osmotic shock and adaptation display a higher thermotolerance than unexposed cells but the increased thermotolerance could not be directly linked to cell morphology (Jørgensen et al., 1995). Filamentous *Listeria* cells divided into cells of normal size when the osmotic stress was removed (Isom et al., 1995).

Speculation that filamentation may be an adaptative mechanism for survival under adverse condition has not been demonstrated, although the length of filaments increases when the length or severity of the stress is increased (Brandi et al., 1989; Everis and Betts, 2001; Isom et al., 1995). Resistance to freezing and thawing in stationary phase *Lactobacillus acidophilus* grown at 25°C is greater than cells grown at 37°C (Lorca and Valdez, 1998). However, the protective effect attributed to a possible growth rate effect or presence of filamentous cells at 25°C must be interpreted with caution. Filamentation reported to occur at 25°C but not at 37°C was not actually demonstrated and the extent of filamentation in the population is not known (Lorca and Valdez, 1998). The possible effect of growth rate, attributed to fewer cells being at a critical stage of cell division or constriction, in stationary phase cells of *L. acidophilus* may be different than the decreased susceptibility to cold shock observed in log phase *Pseudomonas aeruginosa* growing at lower growth rates (Kenward and Brown, 1978).

Thus filamentation appears to be a general stress response to unfavourable environmental conditions for gram negative and gram positive microorganisms; however, the reason for filament formation is not clear.

1.8 Conclusions

Traditional food processing techniques, such as freezing, blanching, sterilization, curing, and use of preservatives, are being replaced with milder processes because consumer demands for high quality, convenient, less processed and preserved foods are increasing (Abee and Wouters, 1999). Such minimally processed foods and raw meat products rely on refrigeration as the major factor to control the growth of pathogenic and spoilage bacteria (Abee and Wouters, 1999). It is gradually becoming more apparent that bacteria have evolved complex networks to adapt to and survive the various stresses intended to control their growth in foods and may actually develop tolerance to a subsequent exposure or become more resistance or sensitive to other stresses that are not applied at the time of the original stress.

Despite reports of elongation of *E. coli* cells at temperatures just below the minimum for growth nearly 40 years ago, the mechanism is yet to be revealed (Shaw, 1968; Mattick et al., 2000). Filament formation at low temperatures has been attributed to an early block in the cell division process and is most likely related, either directly or indirectly, to the lack of a stable or active FtsZ ring, which is positively or negatively affected by molecular chaperones, compatible solutes, DNA supercoiling, DNA replication and repair, DNA segregation, and the cell division process, all of which are controlled by complex regulatory networks. Increased levels of TF, Hsc66, Hsc20 and trehalose assist the cell to maintain proper protein conformation at low temperatures. DNA topology is influenced by DNA gyrase and H-NS, both of which are positively regulated by CspA. Increased DNA supercoiling increases expression of *proU*, *recA*, and *sulA*. RecA is involved in DNA recombination and repair and is also induced during the SOS response. Proper levels of DnaA must be maintained for proper movement of the replication fork because stalled replication forks lead to the induction of the SOS response. SulA, a cell division inhibitor that binds to FtsZ, is produced during full induction of the SOS response and expression from the *sulA* promoter is also increased at lower temperatures. In addition, FtsZ ring formation is also affected by levels, stability and activity of other cell division proteins, including FtsK produced at higher levels during the SOS response. H-NS is antagonistic towards RpoS but RpoS accumulation at low temperatures is due to increased levels of DsrA that increases the turnover of *hns* mRNA while stabilizing *rpoS* mRNA. Transcription of some the cell division genes, including *bolA* are positively regulated by or independent of RpoS. The round morphology that develops shortly after cells are exposed to environmental stress, due to increased production of BolA, can only occur in the presence of active FtsZ. However, cells become filamentous instead of maintaining the small round morphology after prolonged exposure to environmental stress, which points to a lack of active FtsZ.

This literature review reveals that a detailed knowledge of the behaviour of cold adapted, log phase *E. coli* cells that are exposed to temperatures near the minimum for growth is lacking. However, knowledge of that behaviour is essential for realistic assessments of the risks that may arise from the growth of mesophilic pathogens during commercial processes for the storage and display of food (Gill et al., 1998, 2001). Furthermore, a detailed understanding of the growth response will contribute to the ability to predict microbial growth through development of mathematical models for microbial growth at constant and fluctuating chiller temperatures. Such mathematical models are ideally constructed based on an understanding of the underlying mechanisms of microbial responses to environmental conditions as opposed to the current approach of creating complex equations with numerous parameters in attempts to achieve a better fit to growth curves without having an appreciation for the behaviour of bacteria (McMeekin et al., 1993, 2002). Such models are currently used to assess how temperature during storage and preparation affects the microbiological safety of foods for HACCP purposes, risk assessment, shelf life studies and temperature function integration (McMeekin et al., 1993, 2002). Although the proliferation of pathogens may be slow at temperatures near their minimum for growth, any increase in the numbers of pathogens with very low infectious doses, such as *E. coli* O157:H7, may increase risks to consumers' health (Willshaw et al., 1994).

1.9 Research Objectives

Temperatures of chilled foods may fluctuate above and below the minimum growth temperature of *E. coli* and other related pathogens; however there is a lack of knowledge of the growth response of cold adapted bacteria exposed to temperatures around the minimum for growth. The current assumption is that growth of *E. coli* does not occur below 7ºC. However, a recent study of cold adapted, log phase *E. coli* exposed to temperatures below 7°C indicated that the behaviour was complex and variable with the chiller temperature and data for numbers of cfu and absorbance values for the same broth cultures were contradictory (Gill et al., 2001). The objectives of this research were to gain a better understanding of the behaviour of log phase *E. coli* cells exposed to constant temperatures below and near the minimum for sustained growth and temperatures that fluctuate around the minimum for growth and to determine the physiological response to temperatures below the minimum for growth by monitoring changes in protein expression patterns using two-dimensional gel electrophoresis. An understanding of the behaviour and physiological response of mesophilic pathogens is essential for predicting microbial growth and for identification of effective methods for controlling their growth in chilled foods.

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2. Behaviour of Log Phase *Escherichia coli* at Constant Temperatures Near the Minimum for Sustained Growth

2.1 Introduction

Fabrication of raw meat at temperatures around 10°C may result in *Escherichia coli* and related pathogens being in the logarithmic phase of growth when the product is cooled to chiller temperatures for storage or display (Smith, 1985). Temperatures of meat on retail display may fluctuate above and below the minimum temperatures for growth of *E. coli* and other mesophilic organisms (Olsson, 1990). Although the proliferation of pathogens may be slow at temperatures near their minimum for growth, any increase in the number of cells of a pathogen with a very low infectious dose, such as *E. coli* O157:H7 (Willshaw et al., 1994), may increase risks to consumers' health. Knowledge of the behaviour of cold adapted, log phase cells that are exposed to temperatures near the minimum for growth is therefore required for realistic assessments of the risks that may arise from the growth of mesophilic pathogens during commercial processes for the storage and display of meat (Gill et al., 1998).

Models for predicting the growth of bacteria in foods have been developed by monitoring changes in the optical absorbances of or the numbers of colony forming units (cfu) recoverable from liquid cultures (Adams et al., 1991; Buchanan and Phillips, 1990; Gibson et al., 1988). When models that relate the durations of lag phases and the growth rates of bacteria to the physical and chemical conditions of growth media are constructed from such data, it is necessarily assumed that the population of cells in each culture is homogeneous (Baranyi et al., 1993). While that may often be the case, the assumption is not always warranted (Davey and Kell, 1996; McKellar, 1997).

The behaviour of cold adapted, log phase *E. coli* at temperatures below 7°C, the minimum temperature for growth of this organism, was recently examined (Gill et al., 2001). The findings of that study were that absorbance of broth cultures incubated at

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nonpermissive temperatures increased at declining rates for several days, but no corresponding increases in numbers of cfu as determined by plating were apparent. When *E. coli* cultures were returned from 2°C to a growth permitting temperature, the duration of the lag phase apparently increased, and initial growth rates apparently decreased with increasing times of incubation at 2°C. The behaviour of log phase *E. coli* exposed to chiller temperatures appeared to be complex and variable with the chiller temperature, while data for numbers of cfu and absorbance values for the same broth cultures were contradictory. The increases in absorbance values may have been due to the elongation of cells to form filaments, which was observed in log phase *E. coli* cells incubated at 6°C (Shaw, 1968).

Obviously, models based on either absorbance or plate count data from such cultures, with the assumption of culture homogeneity, could not accurately describe the behaviour of *E. coli* at temperatures that fluctuate about the minimum for growth (Gill et al., 2001). The potential for elongation of *E. coli* cells at temperatures a few degrees below the minimum for growth raises the possibility that elongation may occur also at temperatures above the minimum for growth. If that should be the case, then current models which relate the growth of *E. coli* to temperature may be inaccurate for temperatures that approach 7°C. The matter is of some practical concern because these models are used in the assessments of how temperature during preparation and storage affect the microbiological safety of foods, such as raw meats, in which the growth of enteric pathogens is controlled by temperature alone (Cassin et al., 1998). Therefore, the behaviour of *E. coli* at temperatures below and near to but above the minimum for growth was examined in detail to resolve the discrepancies between numbers of cfu and absorbance data for the same cultures, and to better characterize the lag phase that develops in log phase *E. coli* exposed to chiller temperatures.

2.2 Materials and Methods

2.2.1 Culture preparation and enumeration of E. coli

A wild-type strain of *E. coli* that was isolated from a beef packing plant and maintained in Cooked Meat Medium (Difco Laboratories, BD Diagnostics, Sparks, MD) was cultivated in half strength Brain Heart Infusion (BHI; Difco) for all experiments. The wild-type strain was used for all experiments, except when noted. *E. coli* ATCC 11775 and *E. coli* O157:H7 ATCC 43895, each maintained in Cooked Meat Medium and cultivated in half strength BHI broth, were used to confirm that the behaviour of other *E. coli* strains was similar to that of the wild-type strain. Stock cultures of the *E. coli* strains were stored at -80°C in half strength BHI broth containing 20% (vol vol^{-1}) glycerol.

Cold adapted, log phase cultures were prepared by inoculating 125 ml flasks containing 70 ml of BHI with 2 ml portions of cultures grown to the stationary phase at 25°C, and incubating flasks at 12°C with shaking at 100 rpm for 24 h until an absorbance at 600 nm (A_{600}) of 0.4 to 0.5 was reached. Absorbance values were determined using a spectrophotometer (UltraSpec III, Pharmacia LKB Biotechnology, Uppsala, Sweden). The cold adapted, log phase cultures were used to inoculate flasks of BHI that were prechilled to the temperature at which each flask would be incubated. Numbers of cfu were determined by preparing serial 10-fold dilutions in 0.1% peptone water that were held on ice until a 0.1 ml portion of each appropriate dilution was spread on each of 5 plates of Plate Count agar (PCA; Difco) and on 5 plates of Violet Red Bile agar (VRBA; Difco) for determination of viable and uninjured cells, respectively. PCA and VRBA plates were incubated at 35°C for 24 h. At each sampling time, 1 ml of each culture was mixed with 100 µl of a 37% formaldehyde solution (Fisher Scientific, Edmonton, AB) in a sterile Eppendorf centrifuge tube. The fixed cell preparations were stored at 2°C until required for measurements of cell lengths or flow cytometry.

2.2.2 Survival and growth of cold adapted E. coli *in BHI at temperatures below 7°C and subsequent growth at 12°C*

Eight flasks of BHI were each inoculated with a cold adapted, log phase culture to obtain an initial A_{600} of 0.2. Pairs of flasks were incubated at 2, 3, 5 or 6 $\pm$ 0.1°C in a shaking water bath for 8 days. Portions of each culture were removed immediately after inoculation and at daily intervals for determination of A_{600} values, measurement of cell lengths and the enumeration of colonies on PCA and VRBA.

To determine the behaviour of cultures at 12°C after incubation at temperatures below 7°C, flasks of BHI were each inoculated to obtain an initial A_{600} of about 0.05. Seven pairs of flasks were prepared and incubated, then a further seven were prepared 12 h later to allow cultures to be sampled at convenient times. At each time, one pair of flasks was incubated at 2°C for 4 d, 3°C for 8 d or 5°C for 8 d and three pairs of flasks were incubated at 6°C, with one pair being incubated for 0.5 d, 4 d, or 8 d. After incubation at temperatures below 7°C, flasks were incubated at 12°C for 48 h. A pair of flasks prepared at each time was incubated at 12°C to serve as a control. Immediately after inoculation, at the time of transfer to 12°C, and at appropriate intervals during incubation at 12°C, portions of each culture were removed for determination of A_{600} values, measurement of cell lengths, and enumeration of colonies on PCA and VRBA.

2.2.3 Growth of subpopulations of cold adapted E. coli *cells, enriched for normal or elongated cells formed during incubation at 6°C, at 12°C*

A 1000 ml flask containing 300 ml of BHI, inoculated with a cold adapted, log phase culture to obtain an initial A_{600} of about 0.1, was incubated at 6°C in a shaking waterbath for 6 days. A second flask was prepared and incubated 12 h later to allow cultures to be sampled at convenient times during subsequent incubation at 12°C. After incubation at 6°C, the culture and subpopulations of cells were held on ice until the enrichment procedures were completed.

To enrich for cells of normal length, 45 ml of culture in each of six sterile 50 ml centrifuge tubes, was centrifuged at 708 x *g* for 5 min at 6°C. The upper 20 ml from each tube was pooled and divided evenly among three 50 ml centrifuge tubes and the centrifugation procedure was repeated while the pellets were retained for enrichment of elongated cells. The upper 20 ml from each tube was pooled and divided evenly among two 50 ml tubes and the tubes were centrifuged at 1590 x *g* for 6 min. The supernatant was discarded and the pelleted cells were inoculated into fresh BHI precooled to 6°C to an approximate A_{600} of 0.05, of which 70 ml was dispensed into each of a pair of 125 ml flasks.

To enrich for elongated cells, 1 ml of the suspension of combined pellets from the first centrifugation step was layered over 10 ml of 60% standard isotonic Percoll (SIP; vol vol^{-1}) (Linqvist et al., 1997) in each of eight sterile 15 ml polypropylene conical centrifuge tubes (Falcon Blue Max™; Fisher Scientific). SIP, composed of 100 mg peptone and 850 mg of NaCl in 100 ml of Percoll® (Sigma-Aldrich Canada Ltd., Oakville, ON), was diluted with 0.1 % peptone water containing 0.85% NaCl to the desired concentration. The tubes, placed in the position the furthest away from the center in the rotor adapters, were centrifuged at 7520 x *g* for 1.3 min at 6°C. A puncture was created in the bottom of each tube with a heated 21 G needle and approximately 1 ml of cell suspension was collected in a sterile 1.5 ml Eppendorf microcentrifuge tube. The cell suspension was mixed with 0.5 ml of NaCl peptone and centrifuged at 8160 x *g* for 10 min in a microcentrifuge held at 2°C. The pellets were combined into a single microcentrifuge tube, resuspended in 1 ml of BHI and centrifuged at 8160 x *g* for 10 min. The pelleted cells were inoculated into fresh BHI precooled to 6°C to an approximate A_{600} of 0.05, of which 70 ml was dispensed into each of a pair of 125 ml flasks.

For a control, 45 ml of the culture incubated at 6°C for 6 days was centrifuged at 1590 x *g* for 6 min at 6°C. The pelleted cells were inoculated into fresh BHI precooled to 6°C to an approximate A_{600} of 0.05, of which 70 ml was dispensed into each of a pair of 125 ml flasks.

Each pair of flasks was incubated at 12°C for 48 h. Portions of each culture were removed for determination of A_{600} values, enumeration of colonies on PCA and VRBA and fixed for measurement of cell lengths immediately after inoculation and at 4 h intervals during incubation at 12°C.

2.2.4 Survival and growth of E. coli *in BHI at temperatures between 7°C and 15°C*

Eight flasks of BHI were each inoculated with a cold adapted, log phase culture to obtain an initial A_{600} of 0.05. Pairs of flasks were incubated at 7, 8, 9 or 10°C in a shaking water bath for 8 days or until the A_{600} values approached 1.0. Portions of each culture were removed immediately after inoculation and at appropriate intervals for the determination of A_{600} values, the enumeration of colonies on PCA and were fixed for measurements of cell lengths or for flow cytometry.

Three pairs of flasks, each inoculated with a cold adapted, log phase culture of the wild-type strain, strain 11775 or the O157:H7 strain to an A_{600} of about 0.05, were incubated at 9°C in a shaking water bath for 8 days or until the A_{600} values approached 1.0. Portions of each culture were removed immediately after inoculation and at appropriate intervals for the determination of A_{600} values, the enumeration of colonies on PCA and were fixed for measurements of cell lengths or for flow cytometry.

Eight flasks of BHI were each inoculated with 1 ml of a culture grown to the stationary phase at 25°C. Pairs of flasks were incubated at 12, 13, 14 or 15°C in a shaking water bath for up to 8 days. Cultures were subcultured daily to maintain A_{600} values below 0.2. Portions of each culture were fixed immediately after inoculation and at each time of subculture.

2.2.5 Measurement of cell lengths

For each determination of cell lengths, a drop of molten, 2% agar containing 4% formaldehyde or without formaldehyde for fixed cultures, was added to 10 µl of culture on a microscope slide and the preparation was covered with a coverglass. Slides were stored at 2°C for up to 7 days before they were examined. The slides were viewed under phase contrast illumination using a microscope (Axioscope; Zeiss, Jena, Germany) equipped with an 100X oil immersion lens (1.3 N.A.; Plan NeoFluar; Zeiss) connected to a video camera (DXC 930; Sony Corporation, Tokyo, Japan). The lengths of 100 randomly selected cells were measured on images from each slide, using Image Pro-Plus software, Version 4.0 (Media Cybernetics, Silverspring, MD).

2.2.6 Flow cytometry

Fixed cultures were diluted to a density of about 10^6 cells ml^{-1}. The diluent was 1/20th strength BHI supplemented with 8.8 g l^{-1} of NaCl. The diluent was filtered through a 0.2 µm pore size Millipore filter (Fisher Scientific) to remove particles that could interfere with flow cytometry determinations. A 0.5 ml volume of each diluted culture was mixed with 0.5 µl of a nucleic acid stain (SYTO BC bacteria stain; Molecular Probes, Eugene, OR). The mixture was incubated for 5 min. at room temperature. Forward angle light scatter (FALS) and fluorescence data were obtained with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The FALS of a minimum of 5000 cells gated on fluorescence were analyzed with CellQuest software (Becton Dickinson). The 90th percentile for FALS measurements for cold adapted, log phase cells before incubation at temperatures below 10°C was identified as a reference value. The reference value at the 90th percentile was determined after 1 day of incubation for cultures that were incubated above 12°C.

2.2.7 Analysis of data

For each pair of cultures, mean increases in $\log_{10}A_{600}$ values ($\log_{10}(A_{600}$ at $t_{(x)}/A_{600}$ at $t_0))$, where $t_{(x)}$ is the time of incubation in hours and t_0 is 0 h, were plotted against the time of incubation. For cultures incubated at 12°C after being incubated at temperatures below 7°C, the duration of the lag was determined from the intercept with the x-axis of the backward extrapolation of the regression line to the steepest part of the $\log_{10}$ increase of A_{600} vs time curve. Colony counts were converted to $\log_{10}$ values and mean log values were calculated for each set of 5 plates for each culture. The duration of the lag was calculated from the intercept with the log cfu at 0 h of the backward extrapolation of the line to the steepest part of each log cfu vs time curve. The cell lengths were tested for normal distribution using the Wilk-Shapiro test in the PROC UNIVARIATE procedure of SAS (SAS Version 8, SAS Institute, Cary, NC). Mean lengths and the mean length of the longest 10% of cells were calculated using Microsoft Excel 97 (Microsoft Corp., Redmond, WA).

2.3 Results

2.3.1 Behaviour of cold adapted E. coli *at temperatures below 7°C*

The A_{600} values of cultures incubated at 2°C for 8 days remained constant (Fig. 2.1). At 3°C or 6°C, the A_{600} values increased slightly for 3 days or at a progressively diminishing rate for 5 days, respectively, then slowly declined (Fig. 2.1; Table 2.1). During the incubation of cultures at 2°C, the numbers of colonies recovered on PCA declined progressively (Fig. 2.2). In contrast, when cultures were incubated at 6°C, the numbers of colonies recovered on PCA increased during the first day, but subsequently decreased slowly. During incubation at 3°C or 5°C, the numbers of colonies recovered on PCA decreased slightly or remained constant, respectively (Table 2.1.) During incubation at 2°C or 6°C, the number of colonies recovered on VRBA were about 10% or between 60 and 80%, respectively, of the numbers of colonies recovered on PCA (Fig.2.2). The standard deviations within sets of log counts were < 0.1 or < 0.2 log unit

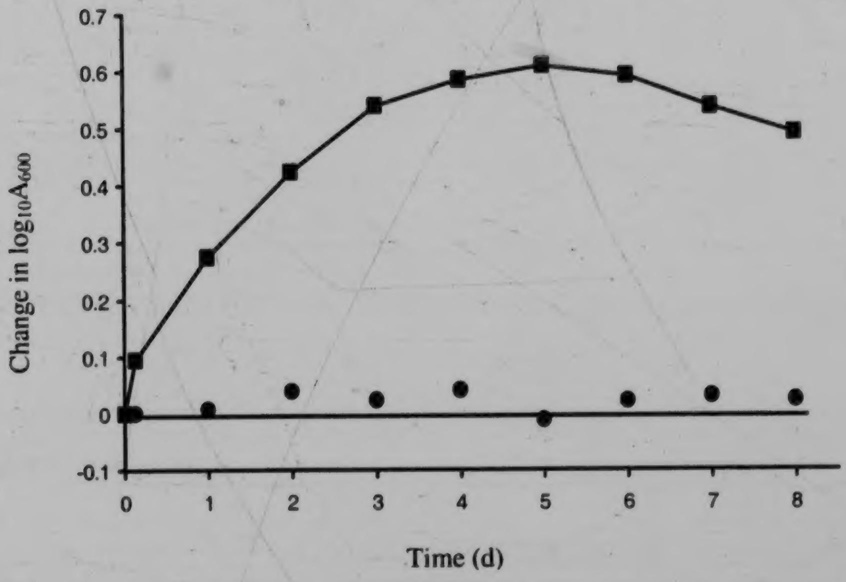

Figure 2.1. Changes in absorbance values [$\log_{10}(A_{600})$] of cold adapted, log phase *E. coli* cultures incubated at 2°C (●) or 6°C (■) for 8 days.

Table 2.1. Behaviour of cold adapted, log phase *E. coli* cultures incubated at temperatures < 7°C for 8 days and after subsequent incubation at 12°C.

Parameter	Temperature (°C)			
	2	3	5	6
Change in log A_{600}	0	<0.1	0.3	0.6
Maximum increase in log A_{600} (days)		3	8	5
Change in log cfu ml^{-1} on PCA	-1.0	-0.4	0	-0.6
Mean cell length (µm)	3.9	6.5	5.7	7.8
Mean length of the longest 10% of cells	6.4	12.7	11.6	17.9
Abnormal cell shapes (blebs, branches)	-	-	+	+
Cell shape during incubation at 12°C after incubation at temperatures <7°C	Normal	Elongation	Elongation	Elongation

$^-$ absence of abnormal cell shapes, $^+$ presence of abnormal cell shapes

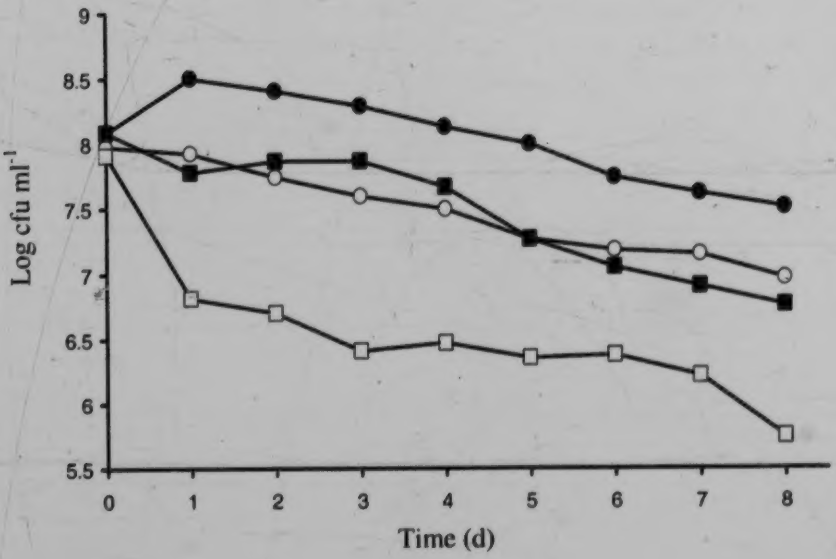

Figure 2.2. Numbers (log cfu ml^{-1}) of *E. coli* recovered on plate count agar (circles) or violet red bile agar (squares) from log phase cultures incubated at 2°C (open symbols) or 6°C (closed symbols) for 8 days.

for colonies recovered on PCA or VRBA, respectively.

During incubation at 2°C (Fig. 2.3), 3°C or 5°C (data not shown), the mean length of the cells and the mean length of the longest 10% of cells remained relatively constant. In contrast, during incubation at 6°C, the mean length of cells decreased during the first day but gradually increased after the third day. The mean length of the longest 10% of cells increased about 3-fold between 1 and 8 days at 6°C. The mean lengths of the shortest 10% of cells were similar at all times at all temperatures.

2.3.2 Behaviour of E. coli *at 12°C after incubation at temperatures below 7°C*

When growth at 12°C was determined from changes in A_{600} values, the exponential growth rate (EGR) of cultures incubated at 12°C was 0.16 generations h^{-1} (Fig. 2.4). For cultures that were incubated at 12°C after being incubated at 2°C for 4 days there was little increase in the A_{600} values during the first 13 h. Thereafter, the growth

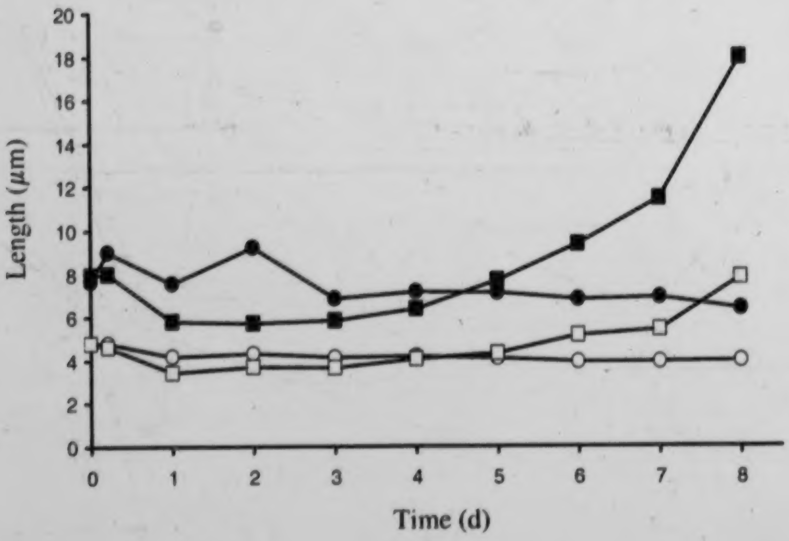

Figure 2.3. Mean lengths of all (open symbols) or the longest 10% (closed symbols) of cells in log phase cultures of *E. coli* incubated at 2°C (circles) or 6°C (squares) for 8 days.

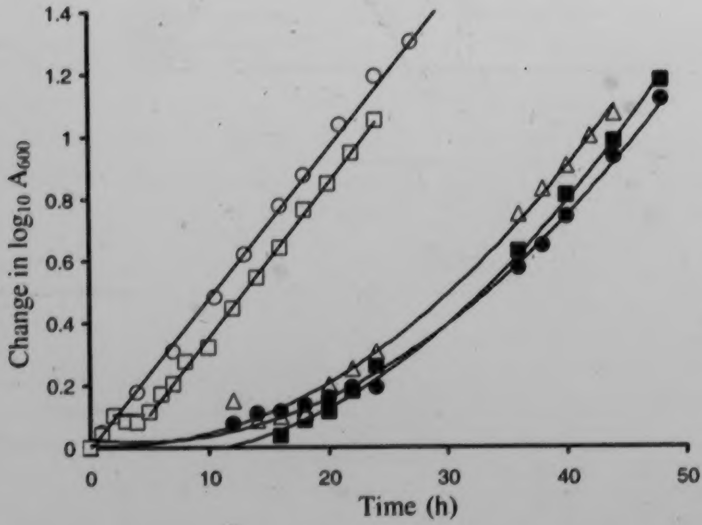

Figure 2.4. Changes in absorbance values [$\log_{10}(A_{600})$] of cold adapted, log phase *E. coli* cultures incubated at 12°C (○) or incubated at 12°C after incubation at 2°C for 4 d (●) or at 6°C for 0.5 d (□), 4 d (■), or 8 d (Δ).

rate gradually increased until the maximum growth rate was attained after 34 h. After that time, the EGR was identical to that of the control culture. Back extrapolation of the log increase of A_{600} vs time curve for times after 34 h yielded an apparent lag time of 24 h. Cultures that were incubated at 6°C for 0.5 or 4 days before being incubated at 12°C had short or long lag durations, respectively, but all attained an EGR of 0.16 generations h^{-1}. The pairs of cultures incubated at 6°C for 8 d before being incubated at 12°C had different lag durations of 15 h or 20.5 h, and different EGRs, of 0.12 or 0.09 generations h^{-1}.

When growth at 12°C was determined from changes in the numbers of colonies recovered on PCA, the EGR of cultures incubated at 12°C was 0.16 generations h^{-1} (Fig. 2.5). For cultures that were incubated at 2°C for 4 days before being incubated at 12°C, an apparent lag of 10 h was followed by an EGR identical to that of the control culture. With cultures that were incubated at 6°C for 0.5 day, the numbers of colonies recovered on PCA had not declined from the numbers recovered before incubation. When such cultures were incubated at 12°C, growth occurred without lag. After that initial growth, growth ceased for 7 h. When growth resumed, the EGR was slightly lower than that of the control culture. The apparent lag was 4 h. Numbers of colonies recovered on PCA decreased in cultures incubated at 6°C for 4 or 8 days. Cultures incubated at 6°C for 4 days before being incubated at 12°C had an apparent lag of 16 h. With such cultures, the initial rate of growth was 0.13 generations h^{-1} between 16 h and 24 h at 12°C. However, between 32 and 36 h the growth rate accelerated to a rate much higher than that of the control culture. A lag and EGR could not be calculated from cultures incubated at 12°C after being incubated at 6°C for 8 days because the culture did not approach a constant rate of growth.

Inoculation of cultures into BHI at 2 or 6°C resulted in differences of 0.6 to 2.0 or 0 to 0.6 log cfu, respectively, between the numbers of colonies recovered on PCA or VRBA (data not shown). Numbers of colonies recovered on VRBA from cultures that were incubated at 2°C for 4 days were $\geq$ 1 log cfu less than numbers recovered on PCA. When such cultures were incubated at 12°C, the numbers of colonies recovered on VRBA decreased during the first 4 h, but the numbers of colonies recovered on PCA remained unchanged (Fig. 2.5). After 24 h at 12°C, the numbers of colonies recovered from VRBA were similar to the numbers recovered on PCA but at 34 , 44 and 48 h, the differences

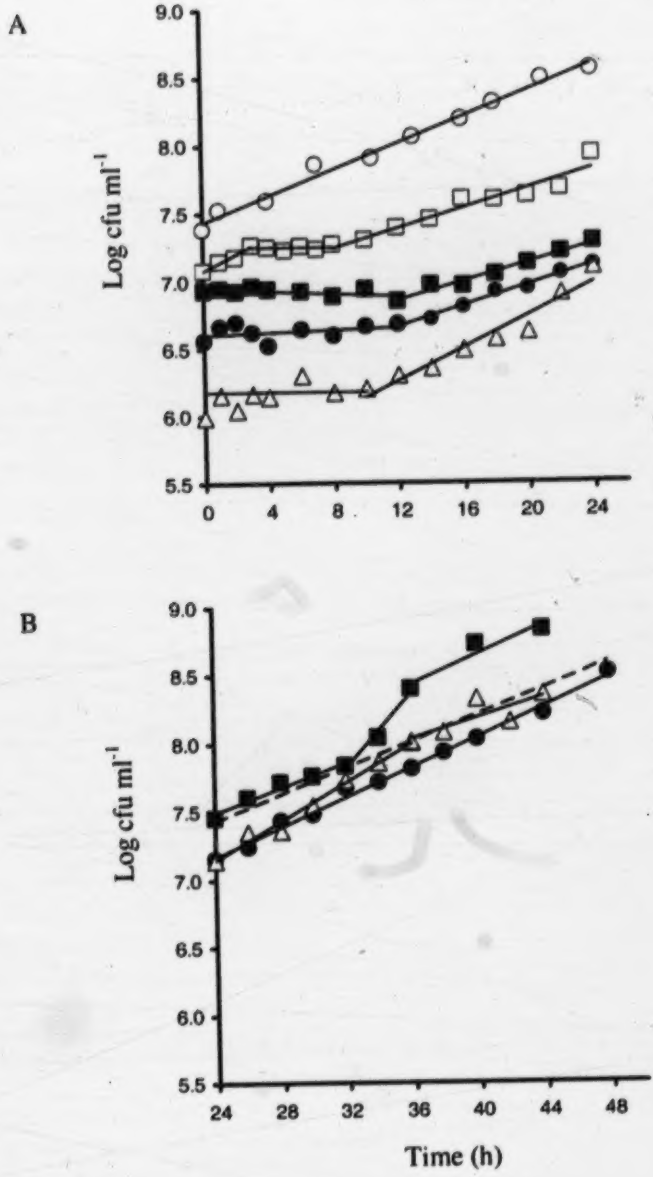

Figure 2.5. Numbers (log cfu ml^{-1}) of *E. coli* recovered on plate count agar from log phase cultures incubated at 12°C (○) or incubated at 12°C after incubation at 2°C for 4 d (●) or at 6°C for 0.5 d (□), 4 d (■), or 8 d (Δ); growth rate of control culture (- - -). Incubation at 12°C between 0 and 24 h (A), or between 24 and 48 h (B).

between the numbers of colonies recovered on VRBA and PCA were about 1 log cfu. Numbers recovered on VRBA from cultures that were incubated at 6°C for 0.5 or 4 days were less than the numbers recovered from PCA. After 18 h at 12°C, the numbers recovered from VRBA were similar to the numbers recovered on PCA. Between 24 and 48 h at 12°C, the numbers recovered from VRBA were less than the numbers recovered from PCA. Numbers of colonies recovered on VRBA from cultures that were incubated at 6°C for 8 days were somewhat less than the numbers recovered on PCA (Fig. 2.6). Differences between the numbers of colonies recovered on PCA and VRBA fluctuated, with maximum differences at 6 h intervals, during incubation at 12°C for times beyond 18 h. The standard deviations within sets of log counts were about 0.1 log unit or ranged up to 0.6 log unit for colonies recovered on PCA or VRBA, respectively, for cultures incubated at 12°C after incubation at 2°C for 4 d or at 6°C for 0.5, 4 or 8 days.

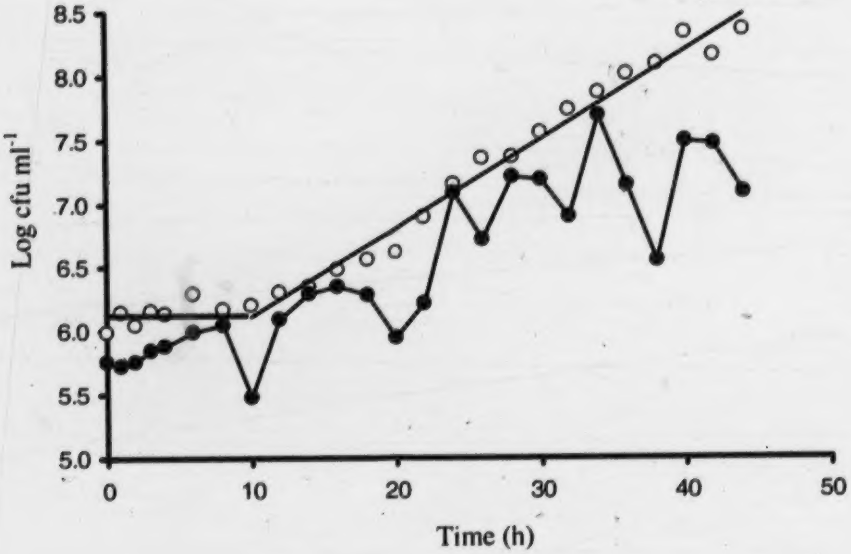

Figure 2.6. Numbers (log cfu ml^{-1}) of *E. coli* recovered on plate count agar (○) or violet red bile agar (●) from log phase cultures incubated at 12°C for 48 h after incubation at 6°C for 8 days.

The mean lengths of cells for cultures incubated at 12ºC and for cultures incubated at 2ºC for 4 days before being incubated at 12ºC, ranged from 4 to 5 µm (Fig. 2.7). The mean lengths of cells for cultures incubated for 0.5 day at 6ºC declined after 1 h at 12ºC and remained below 4 µm for the next 5 h. However, the mean lengths of cells for cultures incubated at 6ºC for 4 or 8 days before being incubated at 12ºC increased with increasing incubation time, to maximum lengths after 26 h. After 44 h at 12ºC, the mean cell lengths had decreased to about 5 µm. The mean lengths of the longest 10% of cells in cultures incubated at 2, 6 or 12° C were ≤ 11 µm (Fig. 2.8), but for cultures incubated at 12ºC after being incubated at 6ºC for 4 or 8 days, the mean lengths of the longest 10% of cell increased with time, to reach maximum lengths of 23 or 40 µm, respectively, after 26 h. The mean lengths and the mean lengths of the longest 10% of cells remained nearly constant for cultures incubated at 3°C or 5°C for 8 days but increased after subsequent incubation at 12°C with increasing incubation time (Table 2.1). The maximum mean length of the longest 10% of cells for cultures incubated at 3°C or 5°C for 8 days before incubation at 12°C reached 26 µm after 32 h or 46 µm after 28 h, respectively (data not shown).

The distribution of cell lengths at any sampling time was not normal. Most cells incubated at 12ºC were < 10 µm long, but most cells incubated at 12ºC for 26 h after being incubated at 6ºC for 8 days were > 10 µm long (Fig. 2.9). Branched and swollen cells were observed with increasing incubation time at 5°C and 6°C but not at lower temperatures (Fig. 2.10). Multiple septa were not observed in elongated cells formed during incubation at 6°C but were observed when cells were subsequently incubated at 12°C (Fig 2.11).

2.3.3 Behaviour of subpopulations of E. coli *cells at 12°C separated by size after incubation at 6°C*

When a culture was incubated at 6°C for 6 days, about 45% of cells gave FALS measurements below the reference value of the 90th percentile of cold adapted cultures before incubation (Fig 2.12). After differential centrifugation, fractions enriched for cells of normal length or elongated cells had about 80% or 25%, respectively, of cells with

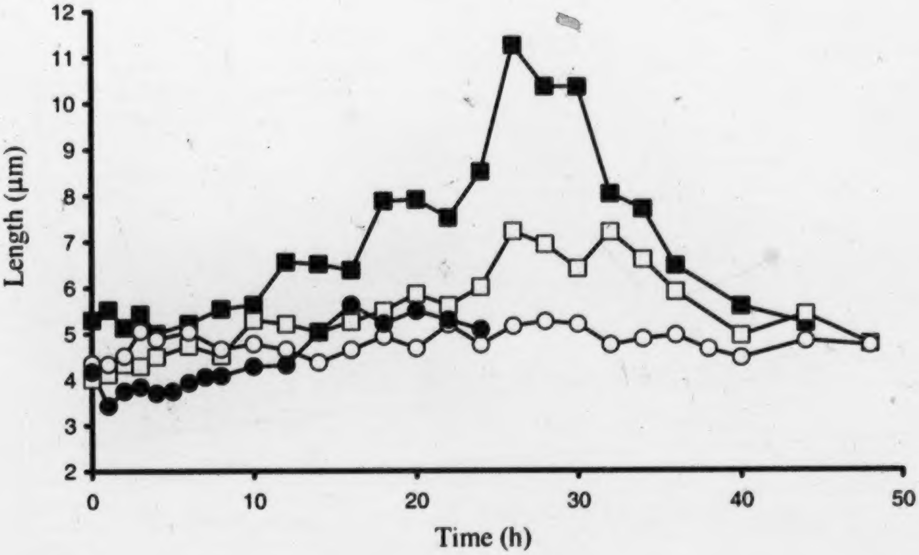

Figure 2.7. Mean lengths of cells in cold adapted, log phase *E. coli* cultures incubated at 12°C for 48 h after incubation at 2°C for 4 d (○) or after incubation at 6°C for 0.5 d (●), 4 d (□), or 8 d (■).

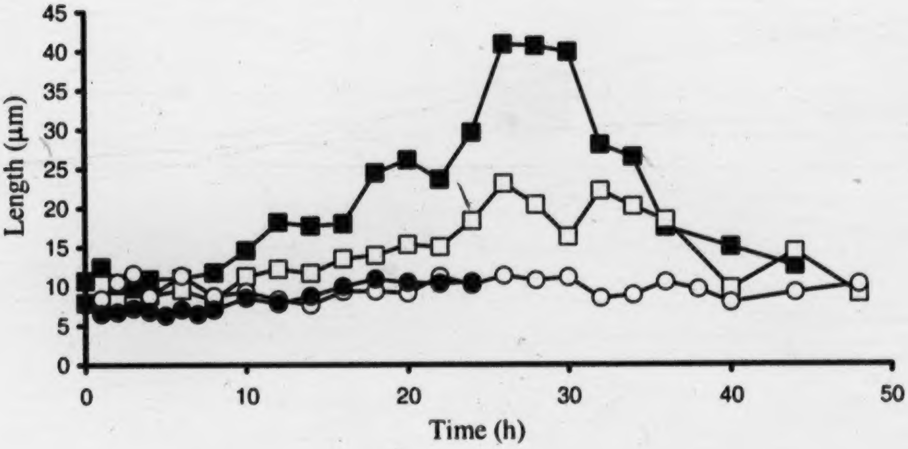

Figure 2.8. Mean lengths of the longest 10% of cells in cold adapted, log phase *E. coli* cultures incubated at 12°C for 48 h after incubation at 2°C for 4 d (○) or after incubation at 6°C for 0.5 d (●), 4 d (□) or 8 d (■).

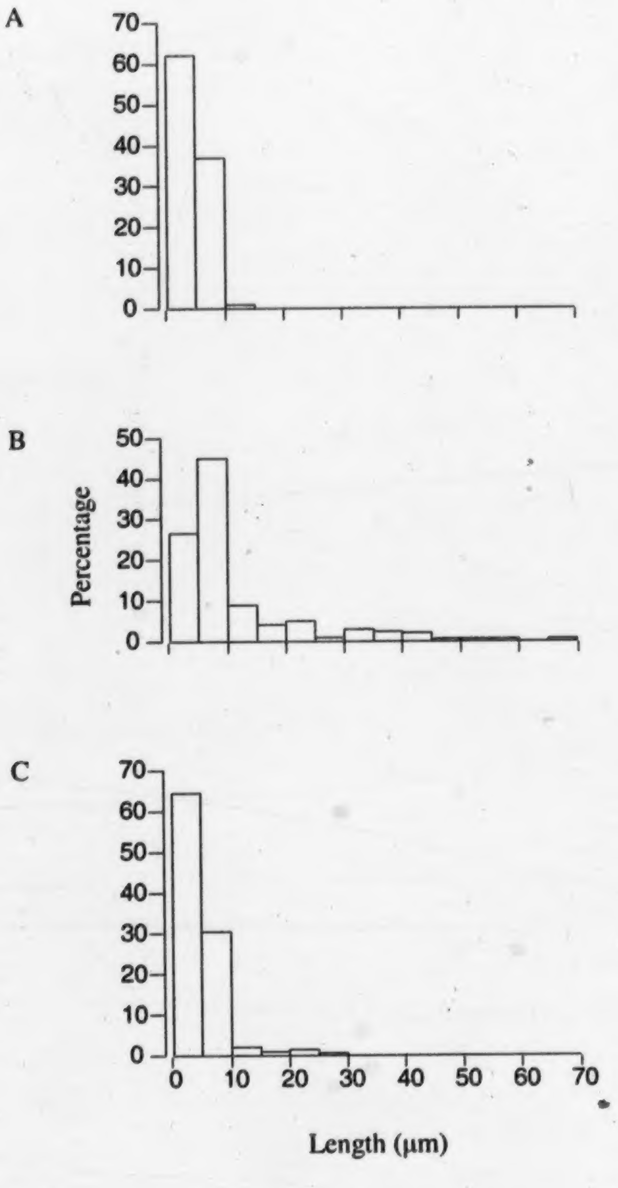

Figure 2.9. Distribution of the lengths of cold adapted, log phase *E. coli* cells incubated at 12°C (control, A), or after 26 h (B) or 44 h at 12°C (C) after being incubated at 6°C for 8 days.

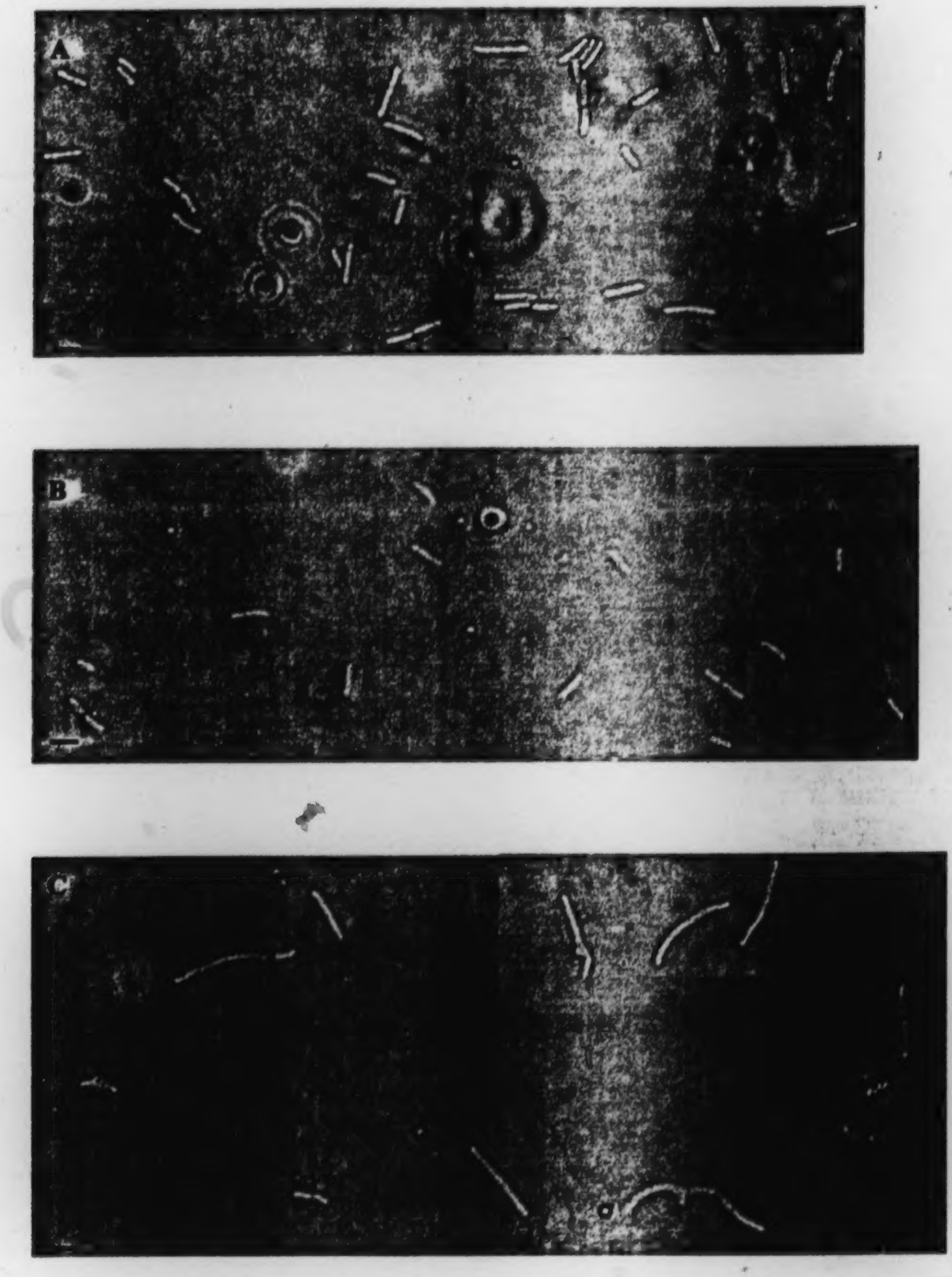

Figure 2.10. Morphology of cold adapted, log phase *E. coli* incubated at 12°C (Control, A), after 8 d at 2°C (B) or after 8 d at 6°C (C); — 2 µm

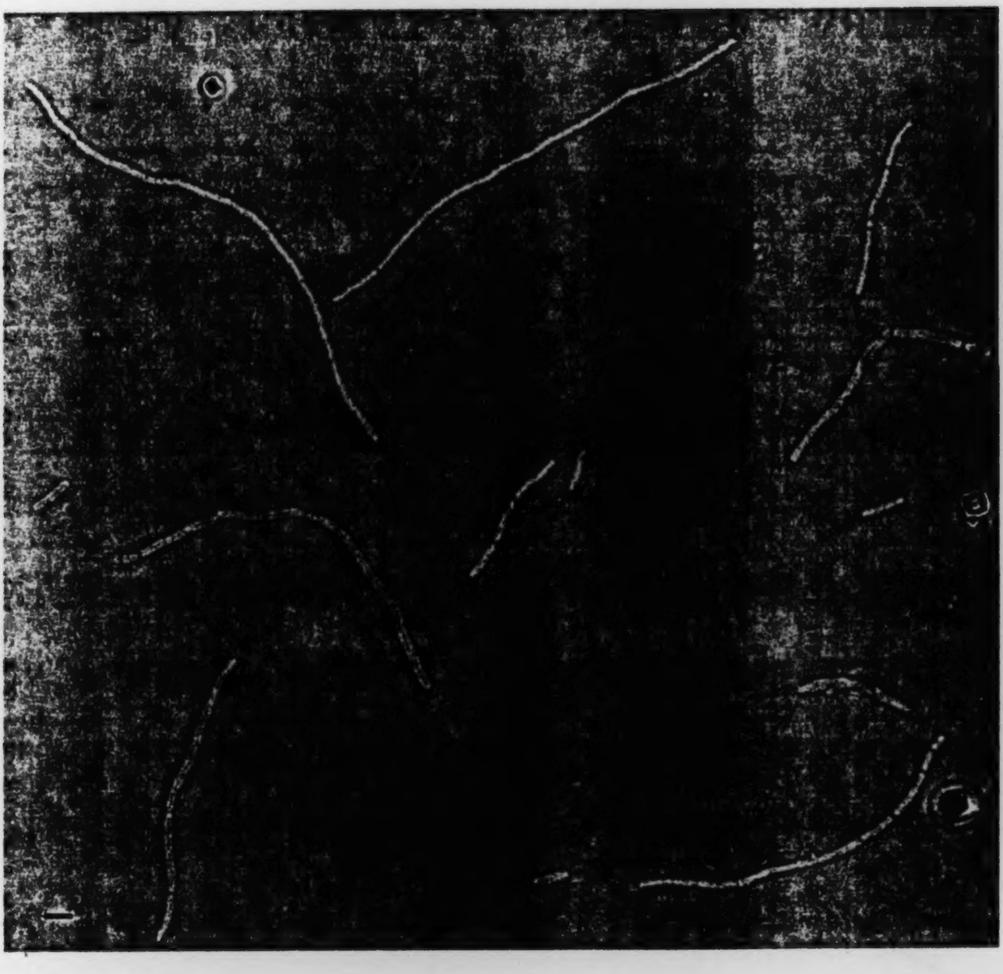

Figure 2.11. Morphology of cold adapted, log phase *E. coli* after 26 h at 12°C after being incubated at 6°C for 8 days; — 2 μm

FALS measurements below the reference value.

When the fraction enriched for cells of normal length was incubated at 12°C, the fraction of cells giving FALS measurements below the reference value gradually decreased to about 60% after 20 h and then gradually increased to more than 90% after 48 h at 12°C. In the subpopulation enriched for elongated cells, the FALS measurements decreased slightly for the first 12 h and then increased to more than 90 % after 48 h.

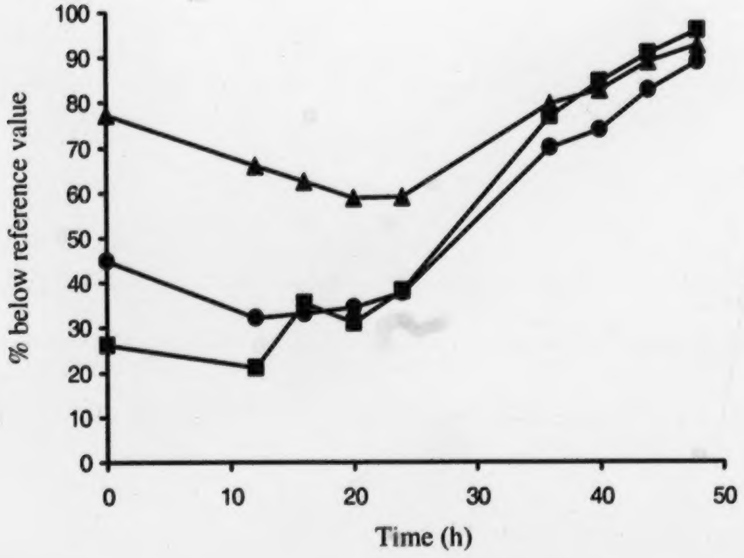

Figure 2.12. Percentage of cells giving mean forward angle light scatter measurements less than the reference value of the 90% percentile before incubation at 6°C during subsequent incubation at 12°C in cultures enriched for cells of normal length (▲) or elongated cells (■) formed after 6 d of incubation at 6°, unseparated (control) culture (●).

For fractions enriched for elongated or cells of normal length, the mean FALS measurements increased by about 50% after 12 h or nearly doubled after 20 h at 12°C, respectively, and then decreased to values below initial FALS measurements (Fig 2.13). The mean FALS measurements of the longest 10% of cells doubled after 20 h at 12°C and then decreased to values 2-fold lower than initial FALS measurements for both subpopulations enriched for elongated or cells of normal length.

The increase in absorbance values for fractions enriched for cells of normal length or elongated cells were similar to the control culture (Fig 2.14). The numbers of cfu increased by about 2.3 log units in the control culture and the culture enriched for cells of normal length and by about 2.8 log units in the culture enriched for elongated cells (Fig 2.15).

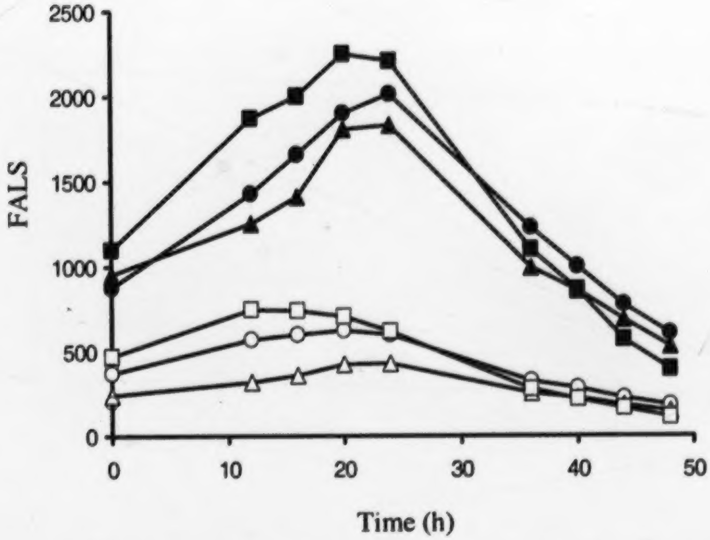

Figure 2.13. Mean forward angle light scatter (FALS) measurements (open symbols) and FALS measurements of the longest 10% of cells (closed symbols) during incubation at 12°C of cultures enriched for cells of normal length (triangles) or elongated cells (squares) formed after 6 d of incubation at 6°, unseparated (control) culture (circles).

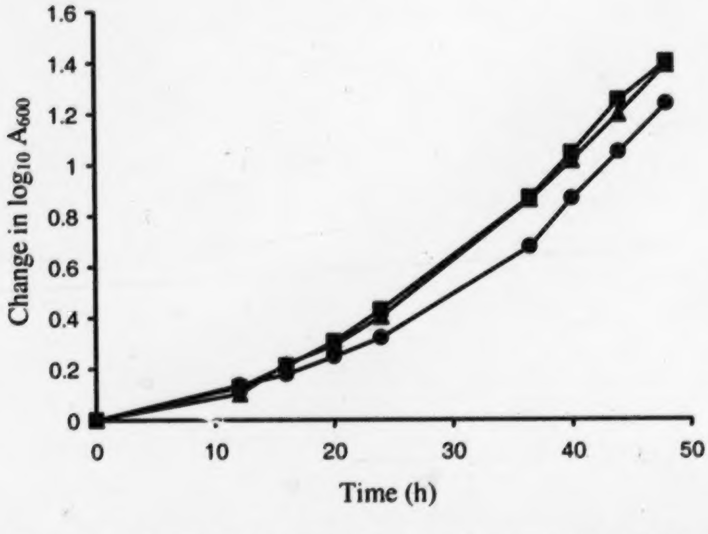

Figure 2.14. Changes in absorbance values [$\log_{10}(A_{600})$] of cold adapted, log phase *E. coli* cultures incubated at 12°C of cultures enriched for cells of normal length (▲) or elongated cells (■) formed after 6 d of incubation at 6°, unseparated (control) culture (●).

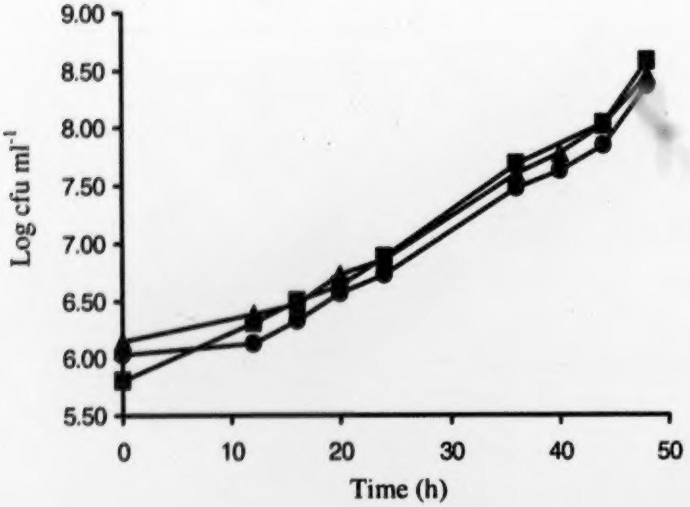

Figure 2.15. Numbers (log cfu ml^{-1}) of *E. coli* recovered on plate count agar from log phase cultures incubated at 12°C of cultures enriched for cells of normal length (▲) or elongated cells (■) formed after 6 d of incubation at 6°, unseparated (control) culture (●).

2.3.4 Behaviour of cold-adapted E. coli *at temperatures between 7°C and 10°C*

When cultures were incubated at 7°C, the numbers of colonies recovered remained constant during the first 3 days and then declined (Fig. 2.16). When cultures were incubated at ≥ 8°C, the numbers of colonies recovered increased with time. At all temperatures the A_{600} values increased, but at 7°C the maximum A_{600} value was less than the values attained at higher incubation temperatures (Fig. 2.17). The generation time estimated from A_{600} values at 10°C was similar to that estimated from numbers of cfu recovered at 10°C but the generation time estimated from A_{600} values at 8°C was more than double that estimated from numbers of cfu recovered at 8°C (Table 2.2).

The mean lengths of cells remained constant in cultures that were incubated at 10°C for 1.25 days, but doubled in cultures incubated at 7°C for 8 days (Fig. 2.18). The mean length of the longest 10% of cells increased by about 50% for cultures incubated at 10°C and doubled for cultures incubated at ≤ 9°C. The mean lengths of the shortest 10% of cells fluctuated between 3 and 4 μm for cultures that were incubated at ≥ 8°C but increased to about 5 μm after incubation at 7°C for 5 days (data not shown).

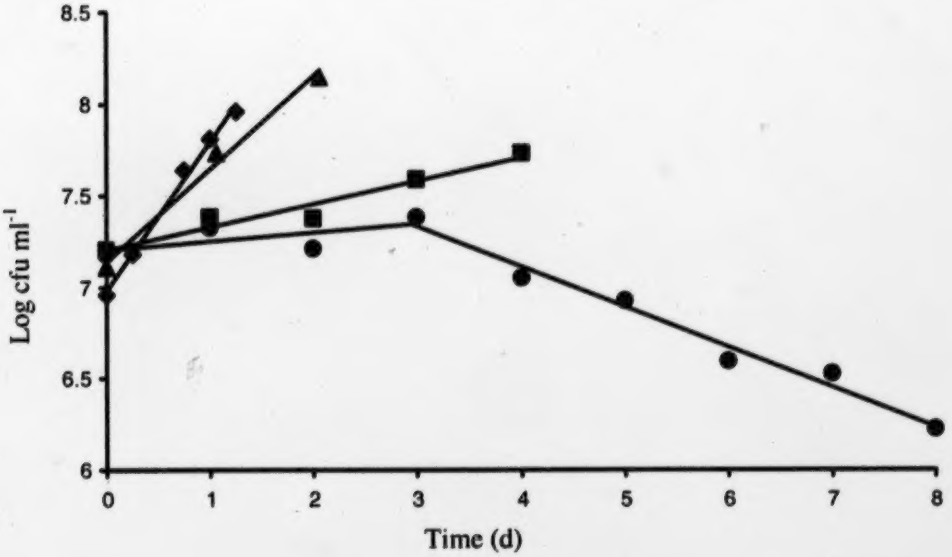

Figure 2.16. Numbers (log cfu ml^{-1}) of *E. coli* recovered on plate count agar from cold adapted, log phase cultures incubated at 7°C (●), 8°C (■), 9°C (▲) or 10°C (◆).

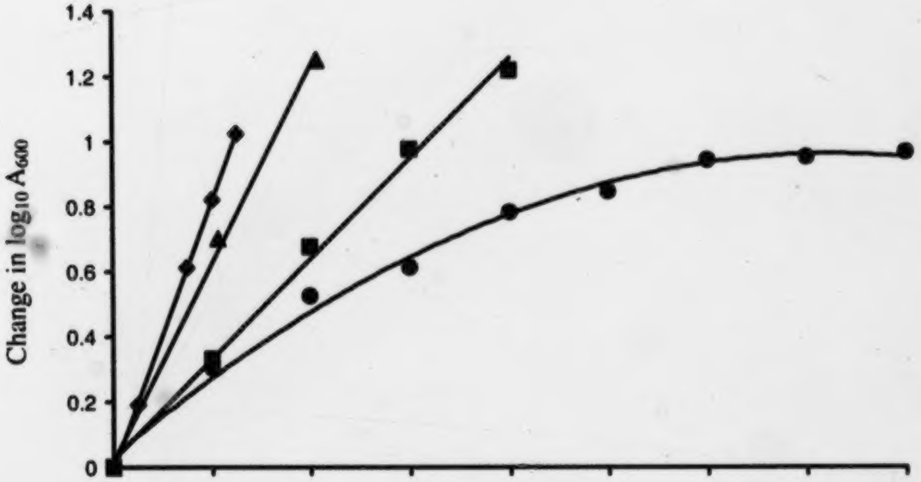

Figure 2.17. Changes in absorbance values [$\log_{10}(A_{600})$] of cold-adapted log phase *E. coli* cultures incubated at 7°C (●), 8°C (■), 9°C (▲) or 10°C (◆).

Table 2.2. Generation times estimated from numbers of cfu recovered and absorbance values from cold-adapted log phase *E. coli* cultures incubated at 8°C, 9°C or 10°C.

Temperature	Generation times (h)	
(°C)	Log cfu	Log A_{600}
8	57.1	23.4
9	14.3	11.8
10	8.8	8.7

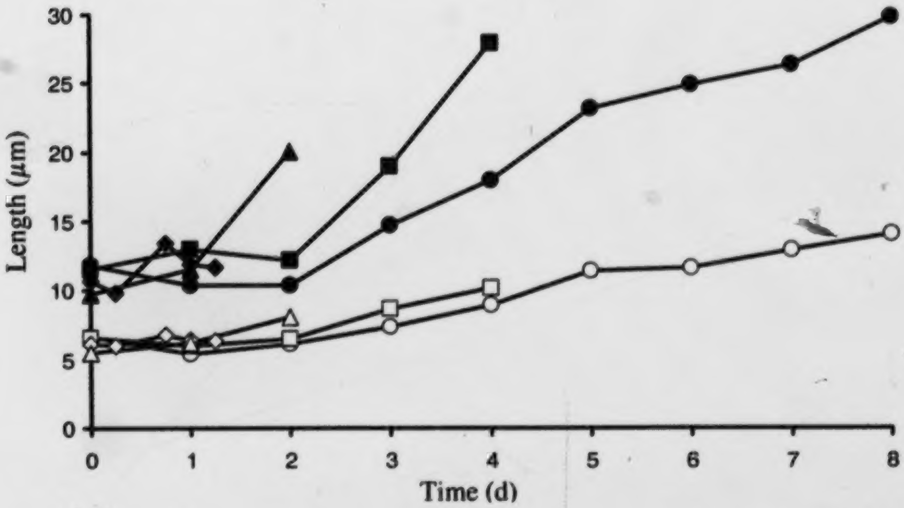

Figure 2.18. Mean lengths (open symbols) and mean lengths of the longest 10% of cells (closed symbols) in cultures of cold-adapted log phase *E. coli* incubated at 7°C (circles), 8°C (squares), 9°C (triangles) or 10°C (diamonds).

For cultures incubated at 7°C for 8 days, the mean FALS value and the mean FALS value of the longest 10% of cells both increased 4-fold (Fig. 2.19). The mean FALS value and the mean FALS value of the longest 10% of cells increased little in cultures that were cultures that were incubated at 8°C for 4 days. The FALS values did

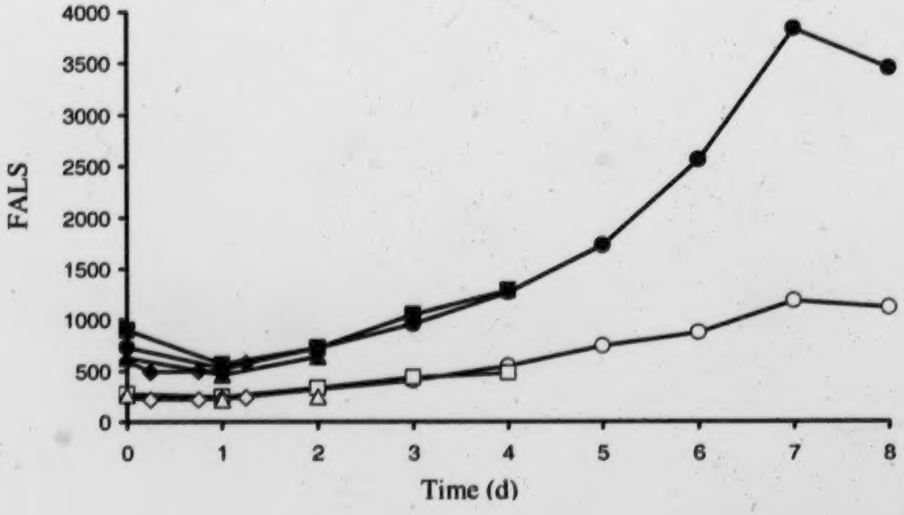

Figure 2.19. Mean forward angle light scatter (FALS) measurements (open symbols) and mean FALS measurements of the longest 10% of cells (closed symbols) of cold-adapted log phase *E. coli* cells incubated at 7°C (circles), 8°C (squares), 9°C (triangles) or 10°C (diamonds).

not increase in cultures incubated at 9 or 10°C for 2 or 1.25 days, respectively. When cultures were incubated at 7°C for 5 days or longer, about 80% of the cells gave FALS measurements above the value for the 90th percentile of cold adapted cultures (Fig. 2.20), but only 17% of cells gave measurements above the reference value when cultures were incubated for 1.25 days at 10°C (Fig. 2.21). The increase in the fraction of cells that gave measurements above the reference value for the 90th percentile of cold adapted cultures was similar for the first three days for cultures that were incubated at 7 or 8°C.

2.3.5 Behaviour of E. coli *between 12°C and 15°C*

The mean lengths of cells increased from about 2.5 µm to about 6 µm during the first day of incubation at all temperatures between 12 and 15°C, inclusive, and remained below 7.5 or 9 µm for cultures incubated at 15°C (data not shown) or 13°C, respectively (Fig. 2.22). The mean lengths of cells and the mean lengths of the longest

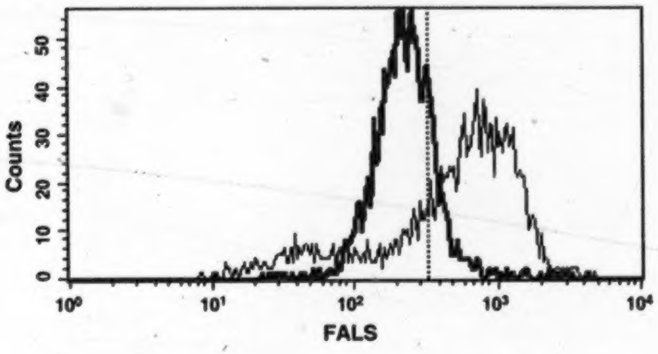

Figure 2.20. Distribution of forward angle light scatter measurements of *E. coli* cells in cold-adapted log phase cultures before (▬) and after 5 days of incubation at 7°C (—), reference value of the 90th percentile before incubation (- - -).

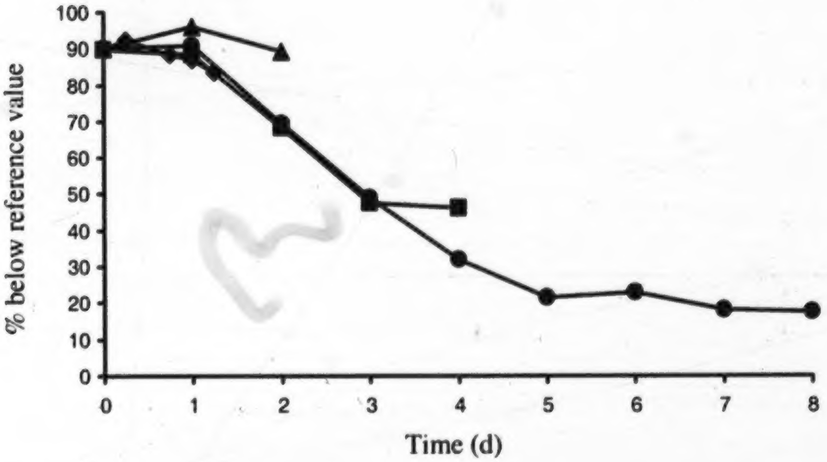

Figure 2.21. Percentage of cold-adapted *E. coli* cells in log phase cultures giving forward angle light scatter measurements below the reference value of the 90th percentile before incubation at 7°C (●), 8°C (■), 9°C (▲) or 10°C (◆).

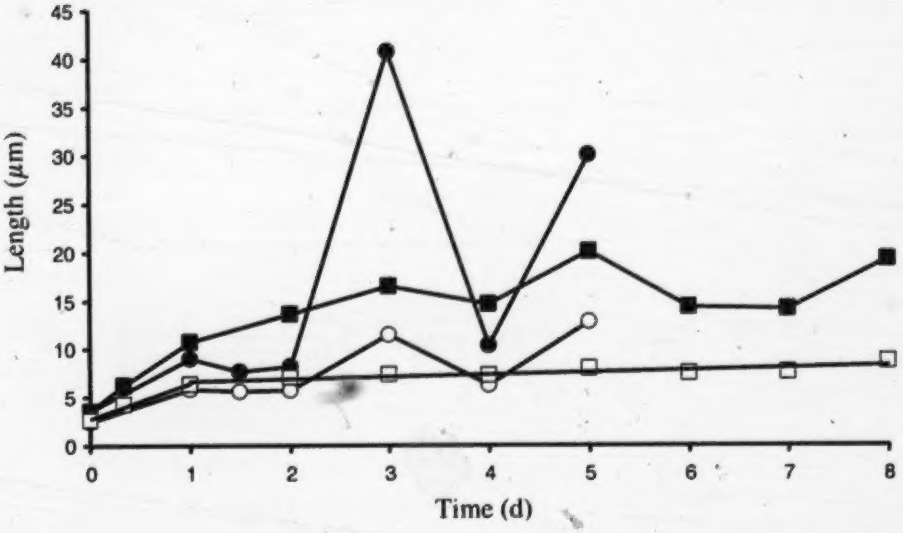

Figure 2.22. Mean lengths (open symbols) and mean lengths of the longest 10% of cells (closed symbols) in cultures of log phase *E. coli* incubated at 12°C (circles) or 13°C (squares).

10% of cells varied considerably during incubation at 12°C. The mean lengths of the longest 10% of cells remained below 20 μm for cultures incubated above 12°C.

The mean FALS values increased 2-fold, from about 100 to about 200, during the first 1 or 1.5 days of incubation at all temperatures. Mean FALS values remained constant for cultures incubated above 12°C, but the values increased gradually to fluctuate between 300 and 400 after 3 days for cultures incubated at 12°C (Fig. 2.23). The mean FALS values for the longest 10% of cells fluctuated between 500 and 700 or between 400 and about 500 after 1 day of incubation for cultures incubated at 13 or 15°C (data not shown), respectively. However, the mean FALS values of the longest 10% of cells increased to 1000 or higher for cultures that were incubated at 12°C for 2 days or longer. When cultures were incubated above 12°C for 8 days, 80 to 90% of the cells gave FALS measurements below the reference value obtained after 24 h of incubation, but for cultures that were incubated at 12°C, 65 to 90% of the cells gave FALS measurements below the reference value (data not shown).

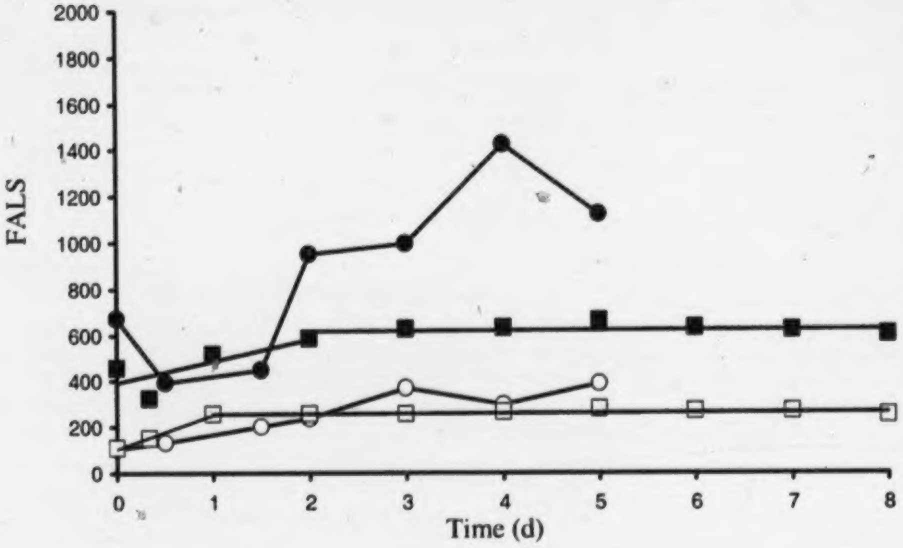

Figure 2.23. Mean forward angle light scatter (FALS) measurements (open symbols) and FALS measurements of the longest 10% of cells (closed symbols) of log phase *E. coli* cells incubated at 12°C (circles) or 13°C (squares).

2.3.6 Behaviour of other cold adapted E. coli *strains at 9°C*

Absorbance values increased in cultures of different *E. coli* strains during 3 days of incubation at 9°C, but increased at greater rates for the wild-type strain than the *E. coli* ATCC 11775 or 43895 strains (Table 2.3). In contrast, the numbers of cfu recovered increased for the wild-type and *E. coli* ATCC 11775 strains but not for ATCC 43895, which is the O157:H7 strain. While >60% of wild-type or *E. coli* ATCC 11775 strains gave FALS measurements below the reference value, only 33% of *E. coli* O157:H7 cells gave FALS measurements below the reference value after 3 days of incubation at 9°C. The mean length of the longest 10% of cells had increased by almost 50% for the wild-type and *E. coli* ATCC 11775 strains but increased almost 3-fold for the *E. coli* O157:H7 strain.

Table 2.3. Behaviour of wild-type, ATCC 11775, and ATCC 43895 strains of *E. coli* after 3 days of incubation at 9°C.

Strain of *E. coli*	Change in $\log A_{600}$	Change in log cfu ml^{-1}	% below the reference value	Relative increase in the mean FALS of the longest 10% of cells
wild-type	1.30	1.21	63.3	1.4
ATCC 11775	0.95	0.86	69.5	1.4
ATCC 43895	0.85	0.03	33.3	2.7

2.4 Discussion

2.4.1 Behaviour of cold adapted, log phase E. coli *at temperatures below 7°C*

In a previous study, when log phase cultures of *E. coli* were incubated at 2ºC, the absorbance values of cultures remained unchanged at incubation times up to 8 days, while the numbers of cfu recovered on PCA or VRBA were similar and did not change (Gill et al., 2001). When log phase cultures were incubated at 6ºC, the A_{600} values increased about 4-fold at declining rates during the first 5 days but changed little thereafter, while the numbers of cfu recovered on PCA or VRBA were again similar and did not change. In this study, the A_{600} values of cultures were unchanged during incubation at 2ºC and increased during the first 5 days of incubation at 6ºC, as in the previous study. However, the numbers of cfu recovered on PCA declined after the first day of incubation at 2ºC, or increased then declined during incubation at 6ºC. Moreover, the numbers of cfu recovered on VRBA were less than those recovered on PCA for all times of incubation at either temperature. The discrepancy between the studies with respect to the numbers of cfu recovered on PCA probably reflects the better discrimination by 5-fold sampling in the second study instead of duplicate sampling (Jarvis, 1989). The discrepancy with respect to the numbers recovered on VRBA possibly reflects differences of inhibitory effects on sublethally injured cells between the batches of the commercial medium used for the two studies (Stephens et al., 1997).

The findings of this study with regard to the numbers of cfu recovered from cultures and the lengths of cells permit a more extensive description of the behaviour of log phase *E. coli* exposed to temperatures of 2 or 6°C than was previously possible. Changes in cell length can be determined by microscopy and by flow cytometry. Although measurements of microscope images give absolute cell lengths and additional detail about cell morphology can be obtained, the number of images that can be conveniently measured in any culture are relatively few. That could result in an incorrect estimation of elongated cell fractions. In contrast, analysis by flow cytometry is rapid but additional information about cell morphology can not be obtained. Therefore, microscopic observations were supplemented by FALS measurements so that relative differences in cell size distributions could be compared by reference to data for many cells in each culture (Durodie et al., 1993). In a flow cytometer, a suspension of bacterial cells stained with a fluorescent dye is injected into the center of a carrier or sheath fluid. The sheath fluid flows slightly faster than the sample, which allows the cells to flow in single file and optically centered past a laser beam (Davey and Kell, 1996). The magnitude of FALS, related to cell length, and right angle or side scatter, related to cell density, as well as fluorescence are detected by photomultiplier detectors and are sorted electronically into bins or channels to allow the display of the intensity of the parameter in histograms or dot plots (Davey and Kell, 1996). In the current study, trends in changes in cell length that were determined by microscopy were supported by FALS measurements.

When log phase *E. coli* were incubated at 2°C growth apparently ceased with little or no delay, as A_{600} values, numbers of cfu recovered on PCA and cell lengths altered little during the first day of incubation at that temperature. However, about 90% of the cells were injured during that time, as indicated by the difference in the numbers of cfu recovered on PCA or VRBA. Subsequently, the numbers of viable cells slowly declined while the fraction of injured cells remained approximately constant. Similar reduction in the numbers of viable organisms during incubation at temperatures below minima for growth of various bacteria have been previously reported (Gill, 2001).

However, when log phase *E. coli* were incubated at 3 or 5°C, A_{600} values increased slightly while numbers of cfu recovered on PCA did not increase during the first day of incubation. Although the mean lengths of cells remained relatively constant during 8 days of incubation, an abnormal morphology was observed for some cells in cultures incubated at 5°C but not in cultures incubated at 3°C.

In contrast, at 6°C, growth continued during the first day, with increases in both A_{600} values and the numbers of cfu recovered on PCA, but with injury of about 50 % of the cells. In addition, the mean length of cells decreased during the first day of incubation, indicating that cells may be switching to a round morphology that has been associated with overproduction of BolA in response to exposure to environmental stress (Aldea et al., 1988). Subsequently, the numbers of cfu recovered on PCA declined while A_{600} values increased. These observations indicate that while a fraction of the cell population was growing, and so increasing the total of viable and moribund cells that were detected by A_{600} measurements, a larger fraction of the cell population was losing viability. The heterogeneous condition of the cultures incubated at 6°C was confirmed by the elongation of a fraction of the cells after 5 days of incubation. The findings disagree with reports of cultures losing viability more rapidly at higher than at lower temperatures below the minimum for growth (Gay and Cerf, 1997; Muntada-Garriga et al., 1995). The discrepancies may be attributed to differences between organisms or to the temperature differential between the test temperature and the minimum growth temperature of each organism.

2.4.2 Behaviour of E. coli *at 12°C after incubation at temperatures below 7°C*

It was previously shown that when *E. coli* is incubated at 2°C for 4 h, a lag of about 2 h at 12°C occurs. After longer times of incubation at 2°C, growth is still initiated after 2 h at 12°C, but an accelerating phase of growth increases in duration with the time of incubation at 2°C. The extended accelerating phases apparent from A_{600} measurements are likely the result of the fractions of injured and moribund cells increasing as incubation at 2°C is extended.

With cultures returned to 12°C after incubation at 6°C for 4 or 8 days, the A_{600} data indicated behaviour similar to that of cultures incubated at 2°C. This is seemingly contradicted by the numbers of cfu recovered on PCA, and by the invariant mean cell length during that time. The cell length data gave no indication that cells which elongated during incubation at 6°C divided at early times after cultures were returned to 12°C. Instead, the data showed that cells elongated when growth was resumed at 12°C. This was confirmed when similar behaviours were observed in a subpopulation of a culture enriched for cells of normal length and the fraction enriched for elongated cells. Furthermore, while the length of cells remained nearly constant in cultures incubated at 3 or 5°C for 8 days, some cells became elongated when cultures were subsequently incubated at 12°C. Thus, cell elongation occurred only in cultures returned to 12°C when A_{600} values increased during the first day of incubation at temperatures below the minimum for growth but normal growth was observed at 12°C when A_{600} values did not increase during the first day of incubation at temperatures below the minimum for growth. When cultures incubated at 6°C for 4 or 8 days were returned to 12°C, the elongated cells divided between 26 and 32 h to give rates of increase of cfu recovered on PCA that exceeded the rate of exponential growth at 12°C. The increases in cell length between 10 and 26 h and the apparently cyclic variations in the numbers of cfu recovered on VRBA compared with the numbers recovered on PCA suggest that growth might have been synchronized when it resumed at 12°C after incubation at 6°C.

2.4.3 Behaviour of cold-adapted E. coli *at temperatures ≥ 7°C*

Cultures that were incubated at 7°C behaved similar to cultures incubated at 6°C; optical absorbances and cell lengths increased while the numbers of colonies recovered were at first unchanged, and then declined. Thus it appears that 7°C is below the minimum temperature for sustained growth of the strain of *E. coli* used in this study. Growth was sustained at 8°C. Despite that, a large fraction of the cells elongated and the relationship between absorbance and numbers of viable cells altered as growth proceeded. As a result, the growth rate estimate from A_{600} values would be more than double the rate that would be estimated from the numbers of cfu. At incubation temperatures of 9°C and above, elongation of cells did not generally occur during the first day of incubation. Consequently, with data collected during that time, growth rates estimated from A_{600} values or numbers of cfu would be similar, as is usually expected

(McMeekin et al., 1993). However, when incubation at temperatures between 9 and 12ºC inclusive was extended beyond 1 day, the cultures became increasingly heterogeneous in cell size. Elongation of *E. coli* cells at low temperatures appears to be a general occurrence because other strains of *E. coli* behave in a similar manner that is most pronounced as temperatures approach the minimum for sustained growth.

In previous (Gill et al., 2001) and present studies, it was assumed that cultures in the logarithmic phase of growth at 12ºC for >24 h would be homogeneous in cell length because cells are reported to adapt to cold temperatures within 4 h (Jones et al., 1987). However, the cold adaptation process is apparently more complex than has been understood, because a subpopulation of the bacterial cells showed distress by elongating after a prolonged period of time at temperatures as high as 12ºC.

The finding that cells elongated during incubation over a wide temperature range contrasts with the suggestion that the growth/no growth interface is very narrow under constraining growth conditions (Tienungoon et al., 2000; McKellar and Lu, 2001). The weakness of the current probability models of the growth/no growth interface is that they are constructed from data obtained by optical absorbance measurements alone. Difficulties are also encountered in the modelling of lag phase durations and bacterial growth when factors such as temperature, pH and a_w approach the limits for growth (Gibson et al., 1988; Buchanan and Phillips, 1990). The lack of fit of bacterial growth data to existing models could be partially due to the formation of elongated cells under conditions of low a_w and starvation as well as at low temperatures (Wainright et al., 1999; Mattick et al., 2000).

Growth by elongation when environmental conditions approach the limits for growth may lead to a misunderstanding of the health risks associated with the number of some pathogens recovered from foods, because each would be detected as a single colony with consequent underestimation of the potential numbers of bacteria to which consumers could be exposed. Although the potential increase in bacterial numbers from elongated cells may be of lesser significance for organisms with high infective doses, even small increases in the numbers of pathogens with very low infectious doses, such as *E. coli* O157:H7, may increase risks to consumers' health (Willshaw et al., 1994). As the findings of this study suggests that the behaviours of pathogens at temperatures near their minimum for growth could be complex, current assessments of microbiological risks associated with chilled foods might be erroneous, irrespective of whether they are based on the predictions of "fail safe" models (Little and Knøchel, 1994) or direct determinations of increases in the numbers of colony forming units recovered from foods.

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3. The Behaviour of Log Phase *Escherichia coli* at Temperatures that Fluctuate about the Minimum for Growth

3.1 Introduction

The temperature experienced by chilled foods is a major factor affecting their microbiological safety. Although a number of models have been shown to successfully predict the growth of various pathogenic and spoilage bacteria at constant temperatures, the predictive power of such models generally becomes uncertain at temperatures approaching the minima for growth (McMeekin et al., 2002). In addition, there is a lack of knowledge about the behaviour of bacteria when temperatures fluctuate above and below their minimum for growth. Because of that lack of knowledge, assessments of the risks that may arise from the growth of mesophilic pathogens in chilled foods during storage and retail display must be uncertain (Cassin et al., 1998).

Escherichia coli and related pathogenic organisms grow exponentially on foods such as raw meats when temperatures remain or rise above 7ºC. The current assumption is that those organisms do not grow below 7ºC (Shaw et al., 1971). Growth or survival of bacteria in foods are commonly determined from counts of colony forming units (cfu) recovered from the food. Growth or survival of bacteria in liquid cultures may be determined similarly, or from increases in optical absorbance values because of the direct relationship between cell numbers and optical absorbance of homogeneous cultures (McMeekin et al., 1993). However, recent studies on the behaviour of *E. coli* at temperatures near to and below as well as above their minimum for growth have shown that at such temperatures cultures become heterogeneous with time, because some but not all cells elongated into filaments (Chapter 2). The numbers of cfu decreased while absorbance values increased for cultures incubated at temperatures ≤ 7° but at temperatures > 7°C, the numbers of cfu and absorbance values increased with time. Even though growth was sustained at 8°C, the growth rate estimated from absorbance values was more than double the rate estimated from numbers of cfu; and when cultures that

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were incubated at 6°C were subsequently incubated at 12°C, the elongated cells further increased in length before they divided into cells of normal size.

The formation of filaments by some log phase *E. coli* at temperatures near but above their minimum for growth would seem to support the suggestion that changes in the physiological states of individual cells could be responsible for difficulties encountered in modelling bacterial growth when conditions are marginal (Baranyi et al., 1995). The elongation of *E. coli* cells at temperatures a few degrees below the minimum for growth raises the possibility that division of filaments may occur or filamentation be enhanced when temperatures rise regularly if, perhaps, briefly from below to above the minimum growth temperature, as commonly occurs during defrosting cycles of retail display cases for chilled foods (James, 1996). Therefore, the behaviour of *E. coli* cultures incubated at temperatures below the minimum for growth with periodic fluctuations to temperatures above the minimum for growth was examined.

3.2 Materials and Methods

3.2.1 Culture conditions

A wild-type strain of *E. coli* that was isolated from a beef packing plant and maintained in cooked meat medium (Difco, BD Diagnostics, Sparks, MD) was cultivated in half strength brain heart infusion (BHI; Difco). A stock culture was stored at -80°C in half strength BHI broth containing 20% (vol vol^{-1}) glycerol.

Cold adapted, log phase cultures were prepared by inoculating 1 L flasks containing 500 ml of BHI with 1 ml portions of cultures grown to the stationary phase at 25°C, then incubating the stirred culture at 15°C for approximately 24 h until the absorbance at 600 nm (A_{600}) was about 0.4. Absorbance values were determined using a spectrophotometer (UltraSpec III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Flasks of BHI tempered to 15°C were inoculated with a cold adapted, log phase culture to obtain an initial A_{600} of 0.05. Flasks were incubated at 2°C, 4°C or 6°C ± 0.1°C in a refrigerated water bath with stirring. For cultures that were exposed to fluctuating temperature cycles, the temperature setting was increased periodically to 10°C ± 0.1°C for 35 min. A single flask was incubated at a constant temperature of 6°C or with fluctuations to 10°C at 24 h, 12 h or 6 h intervals. Pairs of flasks were incubated at a constant temperature of 4°C or 2°C or with fluctuations from 4°C at 6 h intervals or from 2°C at 12 h or 6 h intervals. The temperatures of water baths were recorded at 1 min. intervals.

Portions of each culture were removed immediately after inoculation and at appropriate intervals for the determination of A_{600} values, the enumeration of colonies on Plate Count Agar (PCA; Difco) and cell length determination by flow cytometry. Counts of cfu were determined by preparing serial 10-fold dilutions in 0.1% peptone water that were held on ice until 0.1 ml portions of each appropriate dilution were spread on 5 plates of PCA, which were incubated at 35°C for 24 h.

At each sampling time, 1 ml of each culture was mixed with 100 µl of a 37% (w/v) formaldehyde solution (Fisher Scientific, Edmonton, AB) in a sterile microcentrifuge tube. The fixed cell preparations were stored at 2°C until used for analysis by flow cytometry.

3.2.2 Flow cytometry

Fixed cultures were diluted to obtain a preparation of about 10^6 cells ml^{-1}. The diluent was 1/20th strength BHI supplemented with 8.8 g l^{-1} of NaCl. Prior to use in experiments, the diluent was filtered through a 0.2 µm pore size filter to remove particles that could interfere with flow cytometry determinations. A 0.5 ml volume of each diluted culture was mixed with 0.5 µl of a nucleic acid stain (SYTO BC bacteria stain; Molecular Probes, Eugene, OR). The mixture was incubated for 5 min. at room temperature. Forward angle light scatter (FALS) and fluorescence data were obtained with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The FALS of 10,000 cells gated on fluorescence were analyzed using CellQuest software (Becton Dickinson). The 90th percentile for FALS measurements for cold adapted, log phase cells at the time of initiation of incubation at temperatures at or below 6°C was identified as a reference value.

3.2.3 Analysis of data

For each pair of cultures, mean increases in $\log_{10}A_{600}$ values ($\log_{10}$(A_{600} at $t_{(x)}$/A_{600} at t_0) where $t_{(x)}$ is the time of incubation in hours and t_0 is 0 h) were plotted against the time of incubation. Colony counts were converted to $\log_{10}$ values and mean log values and standard deviations were calculated at each sampling time.

3.3 Results

Cultures incubated with fluctuating temperatures were exposed to temperatures above 7°C for 43 ± 1 min per cycle for all temperature regimes tested. For a culture that was maintained at 6°C, the numbers of cfu initially increased and then subsequently declined by almost 3 log units; but when the temperature fluctuated from 6°C at 12 h intervals, the numbers of cfu remained constant for the first 3 days and then decreased slightly before increasing by > 1 log unit (Fig 3.1). The numbers of cfu initially increased then declined slowly when cultures were maintained at 4°C but the counts decreased more rapidly when the temperature fluctuated at 6 h intervals. When cultures were maintained at 2°C, the numbers of cfu decreased by 2.5 log units, but when the temperature fluctuated at 6 h or 12 h intervals (data not shown) the numbers decreased by only 0.7 or 0.4 log units, respectively. The behaviours of cells incubated at 6°C when the temperatures fluctuated at 24 h or 6 h intervals (data not shown) were similar to those of

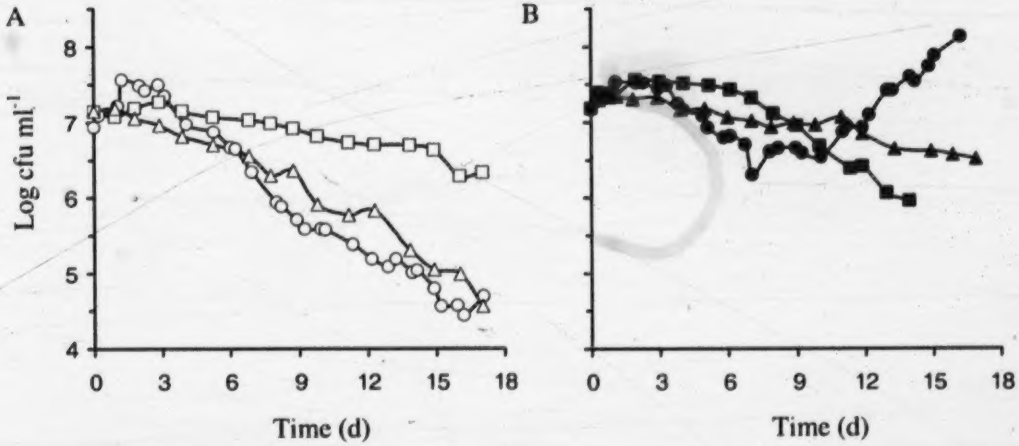

Figure 3.1. Numbers (log cfu ml^{-1}) recovered from cold adapted, log phase *E. coli* cultures incubated at A) constant temperature at 6°C (○), 4°C (□) or 2°C (△) or B) at temperatures fluctuating to 10°C at 12 h intervals from 6°C (●) or at 6 h intervals from 4°C (■) or 2°C (▲).

cells incubated at a constant temperature of 6°C or at temperatures that fluctuated from 6°C at 12 h intervals, respectively. The standard deviation within sets of log counts were < 0.15 log units for all temperature conditions, with the exception when temperatures fluctuated from 2°C at 6 h intervals, where the standard deviation was < 0.3 log units for a single set of log counts.

A maximum increase in log A_{600} values of 0.5 unit was reached after 5 days when cultures were maintained at 6°C, but when temperatures fluctuated from 6°C at 12 h intervals the log A_{600} values increased by 1.2 units (Fig. 3.2). When cultures were maintained at a constant temperature of 4°C the log A_{600} values initially increased and then declined, but when temperatures fluctuated from 4°C at 6 h intervals the log A_{600} values slowly increased. When cultures were incubated at a constant temperature of 2°C, the log A_{600} values remained constant for 3 days and then declined. However, when the temperature fluctuated from 2°C at 6 h intervals, a maximum increase in log A_{600} of about 0.2 unit was attained after 1 day and then maintained.

The mean FALS value of the longest 10% of cells doubled after 9 days and then remained constant when a culture was maintained at a constant temperature of 6°C; but

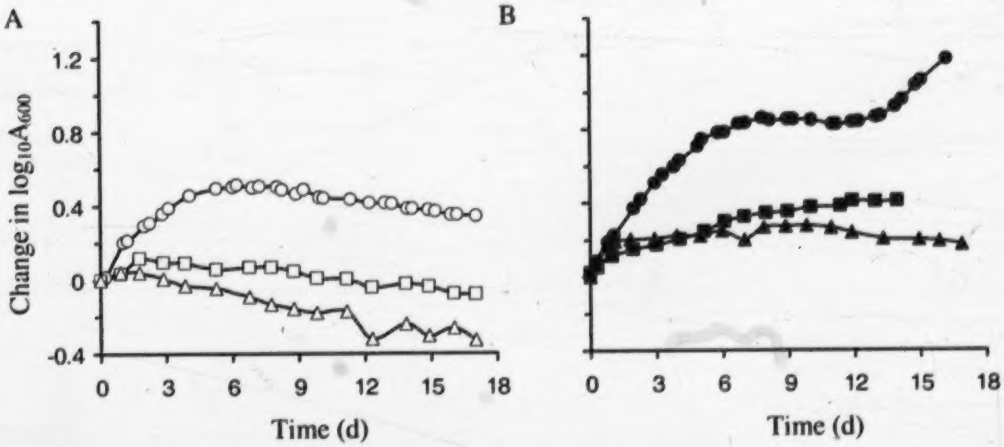

Figure 3.2. Changes in absorbance values for cold adapted, log phase *E. coli* cultures incubated at A) constant temperature at 6°C (○), 4°C (□) or 2°C (△) or B) at temperatures fluctuating to 10°C at 12 h intervals from 6°C (●) or at 6 h intervals from 4°C (■) or 2°C (▲).

when the temperature fluctuated from 6°C at 12 h intervals the mean FALS value of the longest 10% of cells increased 4-fold by 7 days, and then declined within 12 days to values little above initial values (Fig. 3.3). A gradual, 4-fold increase of the mean FALS values for the longest 10% of cells was observed when cultures were incubated at 4°C with fluctuations at 6 h intervals, but the values remained constant for cultures incubated at a constant temperature of 4°C. When the temperature fluctuated from 2°C at 6 h intervals, the mean FALS values of the longest 10% of cells gradually increased 2-fold.

The fraction of cells that gave FALS measurements below the 90th percentile reference value decreased with time and then gradually increased when cultures were incubated at constant or fluctuating temperatures at 6°C and when temperatures fluctuated at 6 h intervals from 4°C or 2°C (Fig. 3.4). The fraction remained relatively constant when cultures were maintained at a constant temperature of 4°C or 2°C or when temperatures fluctuated from 2°C at 12 h intervals (data not shown).

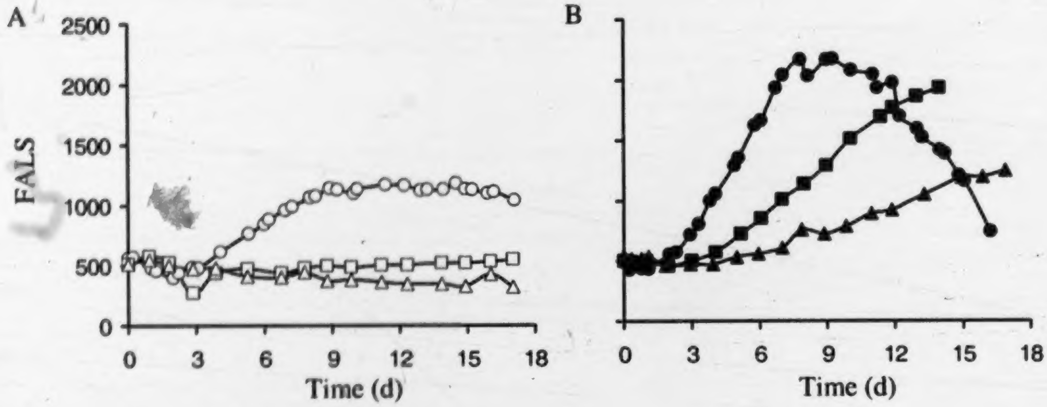

Figure 3.3. Mean forward angle light scatter (FALS) measurements of the longest 10% of cells in cultures of cold adapted, log phase *E. coli* incubated at A) constant temperature at 6°C (O), 4°C (□) or 2°C (△) or B) at temperatures fluctuating to 10°C at 12 h intervals from 6°C (●) or at 6 h intervals from 4°C (■) or 2°C (▲).

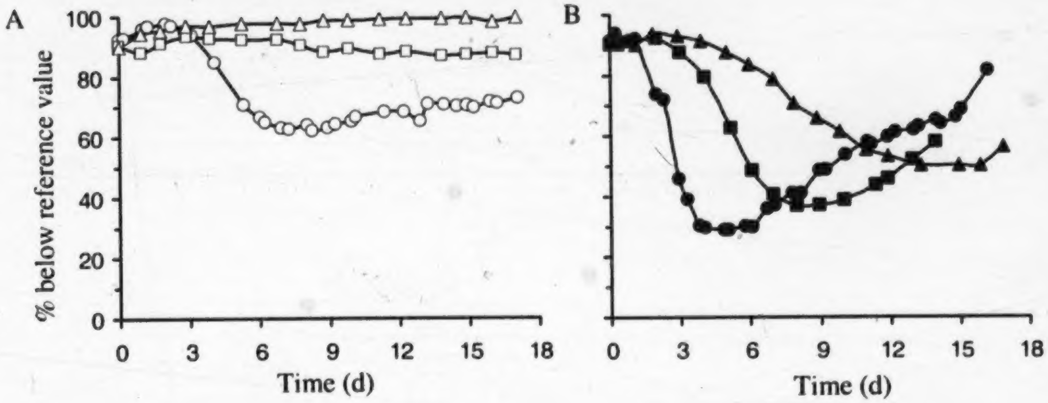

Figure 3.4. Percentage of cold adapted *E. coli* cells in log phase cultures giving mean forward angle light scatter measurements less than the reference value of the 90th percentile before incubation at A) constant temperature at 6°C (○), 4°C (□) or 2°C (△) or B) at temperatures fluctuating to 10°C at 12 h intervals from 6°C (●) or at 6 h intervals from 4°C (■) or 2°C (▲).

3.4 Discussion

The responses of log phase *E. coli* incubated at temperatures < 7°C to brief increases in temperature above 7°C were evidently affected by both the incubation temperature and the frequency of the temperature fluctuations. When cells were incubated at 6°C, temperature fluctuations at 24 h intervals had no obvious effect on the growth or survival of cells.

However, when cells were incubated at 6°C with fluctuations at intervals of $\leq$ 12 h, growth, with elongation of many cells and subsequent division of filaments was apparently sustained. In contrast, when cells were incubated at 4°C, loss of viability was accelerated by temperature fluctuations at 6 h intervals. Despite this, when cells were incubated at 2°C, loss of viability was retarded by temperature fluctuations at 6 h intervals. Some fractions of cells elongated during incubation at 2 and 4°C, and some elongated cells subsequently divided at 4°C when temperatures fluctuated at 6 h intervals, but not when temperatures were constant. This behaviour suggests that responses to the durations of and maximum temperature attained during temperature fluctuations could be similarly varied.

Findings that growth of *E. coli* and gram positive bacteria such as *Brochothrix thermosphacta* under those conditions cannot be accurately predicted by reference to the rate of growth at a constant mean temperature (Li and Torres, 1993; Rajkowski and Marmer, 1995; Gill et al., 1998) could be explained by the complexity of growth responses to temperatures that fluctuate above the minimum for growth. Moreover, the maintenance of growth at temperatures below those generally accepted as the minimum for growth by brief upward fluctuations of temperature must in some circumstances invalidate the predictions of fail safe microbiological models (Little and Knøchel, 1994) and lead to some misassessments of microbiological risks associated with chilled foods. Indeed, the formation of filaments by cells under such conditions may lead to risks being underestimated on the basis of the numbers of cfu recovered from foods, as on warming above chiller temperatures filaments can rapidly divide to substantially increase numbers, apparently without an accompanying increase in biomass (Chapter 2; Mattick et al., 2003). The temperature of 4°C mandated for the storage or display chilled foods (USDA, 1996) may then be inappropriate as a substantial fraction of cells elongated to long filaments at 4°C under temperature fluctuations that are commonly encountered by chilled foods on retail display (Gill et al., 1998). The findings must also increase concern about the large fraction of displayed chilled foods that experience persistent temperatures above 4°C (Gill et al., 2002).

Developing a better understanding of the microbiological risks associated with foods that are exposed to fluctuating temperatures during retail display would seem to be essential if their microbiological safety is to be assured. The findings of this preliminary study that cells behave differently under fluctuating temperatures than under constant temperatures make it obvious that further studies are required to determine the effects of the frequency and durations of as well as the minimum and maximum temperature attained during temperature fluctuations, and to ascertain the extent to which the findings for broth cultures can be used to describe the behaviour of mesophilic pathogens in chilled foods. Moreover, study of the physiological, biochemical, and molecular mechanisms involved in the responses of bacteria to cold temperatures will be required if the growth of pathogenic bacteria in chilled foods is to be accurately predicted and reliably controlled.

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4. Changes in the proteome of *Escherichia coli* in response to temperatures below the minimum for sustained growth

4.1 Introduction

There is a lack of knowledge of the growth response of cold adapted bacteria exposed to temperatures around the minimum for growth. The current assumption is that *Escherichia coli* are unable to grow at temperatures below 7°C. Studies on the behaviour of cold adapted, log phase *E. coli* cultures at temperatures near the minimum for growth indicated that numbers of cfu and absorbance values increased for cultures incubated > 7°C; but at temperatures just below the minimum for growth, numbers of cfu decreased while absorbance values increased with time, due to elongation of a substantial fraction of cells. Absorbance values and cell lengths remained constant during incubation at 2°C (chapters 2 and 3).

Bacteria synthesize all of their cellular components at near constant differential rates and divide at a fixed and constant cell mass when conditions are not restrictive for growth (Neidhardt and VanBogelen, 2000). Bacteria respond to changes in their environment by altering the rate of synthesis of individual proteins, so that new proteins are expressed in response to stress while levels of other proteins decrease (Neidhardt and VanBogelen, 2000). Changes in expressed proteins (proteome) in response to environmental stresses can be monitored. While gene array technology can predict potential proteome modifications by determining changes in transcribed mRNA, changes at the translational or post-translational level are not detected. In contrast, monitoring changes in protein expression profiles permits the direct detection and differentiation of proteins in precursor, mature or modified forms.

Proteomic analysis of cell lysates involves protein separation, detection and identification. Several strategies exist for each step. The classical approach to protein separation relies on gel electrophoresis, which involves movement of charged molecules in an electric field. When protein separation techniques based on molecular weight were combined with protein separation methods based on isoelectric point, resolving power was increased dramatically by the resultant two dimensional procedure (O'Farrel, 1975). Each dimension is capable of resolving approximately 100 to 200 proteins, but when the two techniques are combined, as many as 10,000 proteins can be separated in a large format single gel (Klose and Kobalz, 1995; O'Farrel, 1975). Standard sized gels usually allow the separation of about 2000 proteins (Dunn, 1999). Thus, 2-D gel electrophoresis is a powerful tool for proteomic analysis

In the first dimension, a protein extract is applied to an immobilized pH gradient polyacrylamide gel strip, a current is applied and the proteins migrate to their isoelectric point. A band in an isoelectric focussing (IEF) gel may contain several proteins of different molecular weights that are subsequently separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS is a negatively charged molecule that coats the proteins. When a current is applied in the perpendicular dimension, the coated proteins are separated by size, based on their ability to migrate through the polyacrylamide matrix to the cathode, with smaller proteins moving faster than larger ones (Moat et al., 2002b).

With 2-D gel electrophoresis, proteins can be separated, detected and quantified; and their molecular weights and isoelectric points can be obtained. Some advantages of 2-D gel separation are that cell lysates can be applied directly to the gel and separated with very high resolution, with a minimal loss of hydrophobic proteins (Fichmann and Westermeier, 1999). In addition, isolated proteins embedded in the gel matrix can be readily extracted for further characterization (Fichmann and Westermeier, 1999). Some of the drawbacks of 2-D gel electrophoresis are a lack of reproducibility, because small variations in various steps have a major influence on the results; failure to resolve most proteins > 100 kDa; the inability to separate most membrane proteins; and the need for special gels to resolve extremely acidic or basic proteins (Fichmann and Westermeier, 1999; Mann et al., 2001). Improvements in reproducibility have been obtained by replacing carrier ampholites, which were associated with drifting and distortion problems, with immobilized pH gradient strips in the first dimension (Fichmann and Westermeier, 1999). In addition, multiple gel cast systems and commercially available gels have improved the reproducibility between gels run at different times in the same laboratory or in different laboratories (Fichmann and Westermeier, 1999).

Proteins are invisible on a gel and for detection they must be radioactively labelled during growth or, more commonly, stained after electrophoresis is complete.

Proteins are fixed in the gel to prevent diffusion of the proteins from the gel during staining (Kinter and Sherman, 2000a). Although silver staining is approximately 100-fold more sensitive than staining with Coomassie Blue, silver staining protocols are incompatible with mass spectrometry when glutaraldehyde is used as a fixing and sensitizing agent, because glutaraldehyde crosslinks proteins, which makes them resistant to tryptic digestion (Kinter and Sherman, 2000a; Rabilloud, 1999). Modifications to the silver staining protocols are currently under development to make them compatible with mass spectrometry (Matsui et al., 1999) Another major drawback of silver staining is that colour development is time dependent, which affects the ability to obtain consistent and reproducible results (Kinter and Sherman, 2000a; Rabilloud, 1999). Although Coomassie Blue is not as sensitive as silver stain, it is widely used because of its convenience and adequacy for most electrophoresis applications. However, it is not compatible with immunodetection methods for proteins (Dunn, 1999; Kinter and Sherman, 2000a).

The degree of staining of individual protein spots between different gels is compared with image analysis software. The spots that are differentially expressed are annotated, removed from the gel, and processed for identification. Proteins can be identified by immunologic techniques, such as Western blotting, by amino acid sequencing from the N-terminus, or by mass spectrometry of peptides derived from a tryptic digestion of the protein. Immunologic techniques are dependent on the availability of a suitable antibody, and the confidence of identification is affected by the specificity of the antibody (Kinter and Sherman, 2000b). Mass spectrometry is a highly sensitive and rapid method that generates of large amount of information about peptide fragments that increase confidence in the protein identification (Kinter and Sherman, 2000b). Electrospray ionization and matrix-assisted laser desorption/ionization (MALDI) techniques, developed in the late 1980's, have made mass spectrometry an essential tool for proteomics research (Karas and Hillenkamp, 1988; Mann et al., 2001; Whitehouse et al., 1985).

Peptide fragments, generated by proteolytic cleavage, are ionized under acidic conditions by electrospray or MALDI into protonated gas phase peptide ions that have a distinct mass to charge ratio which is measured by a mass spectrometer. Several types of mass spectrometers have been developed which operate on different principles of mass separation, such as separation based on time of flight (TOF), where the mass to charge ratio is related to the time for an ion to reach the detector; or separation by quadropole electric fields generated by metal rods (quadropole), where a peptide of a certain mass is isolated and selected to pass through the mass filter to reach a detector (Mann et al., 2001). In tandem mass spectrometry (MS/MS), one peptide species is selected out of a mixture in the first mass spectrometer and is then dissociated by collision with an inert gas such as argon or nitrogen. The resulting fragments are separated in the second part of the tandem mass spectrometer to produce a tandem mass spectrum (Mann et al., 2001). The fragmentation patterns of the peptide provide a product ion spectrum that can be matched against databases or interpreted to deduce a partial amino acid sequence from overlapping fragments of the ion spectra (Mann et al., 2001).

For rapid identification of a single protein, a peptide mass fingerprint, usually obtained by MALDI-TOF MS, from a protein digest is compared with the theoretically expected tryptic peptide masses in a database (Mann et al., 2001). To make a match, the protein of interest must be present in the database. In contrast, a mixture of proteins can be identified by tandem mass spectrometry because the peptide mass and a partial peptide sequence can be determined when database searches can match peptides to each corresponding protein (Mann et al., 2001). When the protein is not in a database, the partial sequence may allow the design of appropriate oligonucleotide primers for cloning of the protein's gene using the polymerase chain reaction (Kinter and Sherman, 2000c). Most peptide sequencing is performed on electrosprayed ions because all available basic sites of the peptide tend to be protonated, and therefore give more information about the structure of the ion, whereas ions produced by MALDI tend to be singly charged (Mann et al., 2001). In addition, more ions are produced by electrospray than MALDI, which leads to simpler fragmentation patterns that are easier to interpret (Mann et al., 2001).

Several public databases can be accessed for searching of mass fingerprint and tandem mass spectrometer data. Nonredundant protein databases consist of a known set of complete protein sequences that have been extracted from major sequence repositories where duplicated entries have been removed (Mann et al., 2001). Matches can then be linked to a highly annotated database, such as Swis-Prot (http://expasy.ch/sprot). In addition, genome databases can be searched with mass spectrometric data (Mann et al., 2001). The Genbank database is composed of entries that are directly submitted by individual investigators. It has a high redundancy and a higher rate of error than the much smaller Swiss-Prot database, where entries have been evaluated by a group of scientists before inclusion to obtain a very low redundancy and error rate (Kinter and Sherman, 2000d). Identification of differentially expressed proteins will become simpler with the advancements in 2-D gel technology, mass spectrometry and growth in database entries. Once the identity of a differentially expressed protein is known, the challenge will be to understand its function and regulation.

The objective of this study was to examine the proteomic response of cold adapted, log phase *E. coli* to incubation at temperatures below the minimum for growth, to obtain a better understanding of the physiological changes that occur at temperatures just below the minimum for growth. This knowledge is essential for predicting microbial growth and for identification of effective methods for controlling the growth of bacteria in chilled foods.

4.2 Materials and Methods

4.2.1 Culture conditions

A wild-type strain of *E. coli* that was isolated from a beef packing plant and maintained in Cooked Meat Medium (Difco Laboratories, BD Diagnostics, Sparks, MD) was cultivated in half strength Brain Heart Infusion (BHI; Difco). A stock culture was stored at -80°C in half strength BHI broth containing 20% (vol vol^{-1}) glycerol.

Cold adapted, log phase cultures were prepared by inoculating 1 l flasks containing 500 ml of BHI with 1 ml portions of cultures grown to the stationary phase at 25°C, then incubating the stirred culture at 15°C for approximately 24 h until the optical absorbance at 600 nm (A_{600}) was approximately 0.4. Absorbance values were determined using a spectrophotometer (UltraSpec III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

Two 4 l flasks containing 1.5 l of BHI tempered to 2°C or 6°C were inoculated with a cold adapted, log phase culture to obtain an initial A_{600} of 0.05. Flasks were incubated at 2°C or 6°C ± 0.1°C in a refrigerated water bath, with stirring, for 1 or 8 days, respectively. At each sampling time, the A_{600} was determined, 1 ml of each culture was mixed with 100 µl of a 37% (w vol^{-1}) formaldehyde solution (Fisher Scientific, Edmonton, Alberta) in a sterile Eppendorf centrifuge tube and stored at 2°C until used for analysis by flow cytometry, and 50 ml portions of each culture were mixed with 230 µl of a 37% formaldehyde solution (Fisher Scientific, Edmonton, Alberta) in sterile 50 ml centrifuge tubes, held on ice for 10 min. and centrifuged at 8670 x *g* for 5 min at 2°C. The pellets were combined and resuspended in volumes of ice-cold, sterile, distilled water that were adjusted to give a final cell concentration equivalent to A_{600} values of about 9.0. One ml aliquots of resuspended cells were centrifuged in sterile Eppendorf tubes at 16,000 x *g* for 10 min in a chilled microcentrifuge. The supernatant was discarded and the pellets were stored at -80°C until required for the preparation of protein extracts.

4.2.2 Flow cytometry

Fixed cultures were diluted to obtain a preparation of about 10^6 cells ml^{-1}. The diluent was 1/20th strength BHI supplemented with 8.8 g l^{-1} of NaCl. Before use in experiments, the diluent was filtered through a 0.2 µm pore size filter to remove particles that could interfere with flow cytometry determinations. A 0.5 ml volume of each diluted culture was mixed with 0.5 µl of a nucleic acid stain (SYTO BC bacteria stain; Molecular Probes, Eugene, OR). The mixture was incubated for 5 min. at room temperature. Forward angle light scatter (FALS) and fluorescence data were obtained with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The FALS of 10,000 cells gated on fluorescence were analyzed using CellQuest software (Becton Dickinson). The 90th percentile for FALS measurements for cold adapted, log phase cells at the time of initiation of incubation at temperatures at or below 6°C was identified as a reference value.

4.2.3 Preparation of protein extracts

The preparation of protein extracts, gel electrophoresis, image analysis and excision of protein spots was done by Mike Johns under the supervision of Dr. Austin Murray at Agriculture and Agri-Food Canada (AAFC), Lacombe Research Centre

(Lacombe, AB). Each pellet of cells was washed three times with 250 mM sucrose, resuspended in 44 μl of Milli-Q water and 1 μl of 125 mM pefabloc SC solution (Fluka, Sigma-Aldrich Canada Ltd., Oakville, ON) and subjected to 3 cycles of sonication for 5 sec. The cell mixture was incubated with 5 μl of DNAse/RNAse solution [1mg ml^{-1} DNAse1 (Sigma), 0.25 mg ml^{-1} RNAse A (Sigma), 0.024 M Tris base, 0.476 M Tris-HCl, 0.05 M $MgCl_2$] at room temperature for 1 h, then incubated for a further 1 h after the addition of 450 μl of rehydration buffer [7.7 M urea (Amersham Biosciences, GE Healthcare, Piscataway, New Jersey), 2.2 M thiourea (Fisher Scientific), 2.2% CHAPS (Sigma), 44 mM dithiothreitol (DTT; Amersham), 0.55% IPG pH 4-7 Buffer (Amersham)] and subsequently centrifuged at 21,000 x *g* for 10 min. The protein concentration of the supernatant of each sample was determined (PlusOne 2-D Quant kit, Amersham) and adjusted with rehydration buffer to ensure that each sample had identical concentrations.

4.2.4 Two-dimensional gel electrophoresis

Immobilized pH gradient (IPG) isoelectric focusing (IEF) strips (23 cm, pH 4 to 7), prepared according to the protocol described by Görg (1998) were rehydrated overnight with approximately 900 μg of protein in a 530 μl volume. IEF was carried out in the Multiphor II system (Amersham) for 28 h to achieve a total of 72 kVh. Each IPG strip was equilibrated for 15 min in 10 ml of an equilibration solution composed of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (vol vol^{-1}) glycerol, 2% (w vol^{-1}) SDS (Fisher Scientific) containing 65 mM DTT. Strips were equilibrated for 15 min in 10 ml of equilibration solution containing 135 mM iodoacetamide (Sigma) instead of DTT, and subsequently sealed to the top of a 24 x 24 x 0.1 cm 13.5% SDS-PAGE gel [0.4 M Tris-HCl, pH 8.8, 13.5% acrylamide (Bio-Rad Laboratories, Hercules, CA), 0.1 % SDS, 0.03% ammonium persulfate (Bio-Rad), and 0.03% Temed (Amersham)] using 0.5% agarose (Promega Biosciences Inc, San Luis Obispo, CA) dissolved in SDS electrode buffer. The second dimension protein separation was done using the Ettan Dalt 6 system (Amersham), capable of running 6 gels simultaneously, with a current of 12 W (2.0 W/gel) for the first 45 min followed by a 100 W current for the next 4 h, at 20°C, in electrode buffer that was composed of 25 mM Tris Base, 0.192 mM glycine and 0.1% SDS. The gels were stained overnight in a solution containing 0.05 % Coomassie

Brilliant Blue R-250 (Bio-Rad), 25% (vol vol^{-1}) isopropanol and 10% (vol vol^{-1}) acetic acid; and then destained in a solution containing 10% (vol vol^{-1}) acetic acid and 10% (vol vol^{-1}) isopropanol for a minimum of 5 h in each of three successive baths.

4.2.5 Image Analysis

Images of the gels captured with a digital camera (D1x, Nikon Corporation, Melville, NY) were filtered through a despeckle filter and converted to 16 bit grey-scale images with Photoshop (Adobe, San Jose, CA). Image analysis was done using ImageMaster 5.0 software (Amersham). The volume of the peak at three quarter distance of the height was determined for spots that had different intensities at different sampling times, and the differential expression of the protein spot was determined relative to the volume equivalent of the protein spot on gels prepared using cells from cultures incubated at 15°C. Duplicate gels were produced for each sampling time.

4.2.6 Protein Identification

Differentially expressed spots were excised at AAFC, Lacombe and delivered to the Institute for Biomolecular Design (University of Alberta, Edmonton, AB) for protein identification. An automated in-gel tryptic digestion was performed using a Mass Prep Station (Micromass, Manchester, UK). The gel pieces were de-stained, reduced with DTT, alkylated with iodoacetamide, digested with trypsin (Sequencing Grade Modified, Promega) and the resulting peptides extracted from the gel and analyzed by LC/MS. LC/MS was performed on a CapLC HPLC (Waters, Milford, Massachusetts) coupled with a quadropole-TOF tandem mass spectrometer (Q-ToF-2, Micromass). Tryptic peptides were separated at a flow rate of about 200 to 400 nanol min^{-1} using a linear water/acetonitrile gradient [0.2% (vol vol^{-1}) formic acid] on a picofrit reversed-phase capillary column, (5 μm BioBasic C18, 300 Angstrom pore size, 75 μm ID x 10 cm, 15 μm tip) with an in-line 300 μm ID x 5 mm C18 PepMap column (LC Packings, Sunnyvale, California) used as a loading/desalting column.

Protein identification from the generated MS/MS data was obtained by searching the National Center for Biotechnology Information (NCBI) non-redundant database using Mascot Daemon (Matrix Science, London, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide. Protein matches were searched in the Swiss-Prot protein knowledgebase (Swiss Institute of Bioinformatics, Geneva, Switzerland).

4.3 Results

4.3.1 Behaviour of cold adapted E.coli *cultures at 2 and 6°C*

When a culture growing at 15°C was incubated at 2°C for 1 day, the absorbance values remained constant. When a culture growing at 15°C was incubated at 6°C, the log A_{600} values increased about 0.9 units after 8 days of incubation (Table 4.1). After 4 or 8 days of incubation at 6°C, about two thirds or one third of the cells, respectively, gave FALS measurements below the reference value of the 90th percentile of cultures adapted to the cold by incubation at 15°C prior to incubation at 6°C. The mean length of the longest 10% of cells had not increased after 4 days of incubation at 6°C, but had increased more than 2-fold after 8 days of incubation (Table 4.1).

Table 4.1. Behaviour of cold adapted, log phase *E. coli* after 1 day of incubation at 2°C or after 1, 4, or 8 days of incubation at 6°C.

	Culture conditions			
Parameter	1 d at 2°C	1 d at 6°C	4 d at 6°C	8 d at 6°C
Change in logA_{600}	0.00	0.24	0.64	0.87
% below the reference value	88	93	65	33
Relative increase in the mean FALS of the longest 10% of cells	1.0	0.7	1.0	2.5

4.3.2 Proteomic analysis of cold adapted E.coli *cultures at 2 and 6°C*

Image analysis of the proteomic response of cold adapted *E. coli* cultures before (Fig. 4.1) and after incubation at temperatures below the minimum for sustained growth (Figs 4.2 to 4.5) identified differences in expression for 63 protein spots. When the differentially expressed protein spots were identified by mass spectrometry, the majority

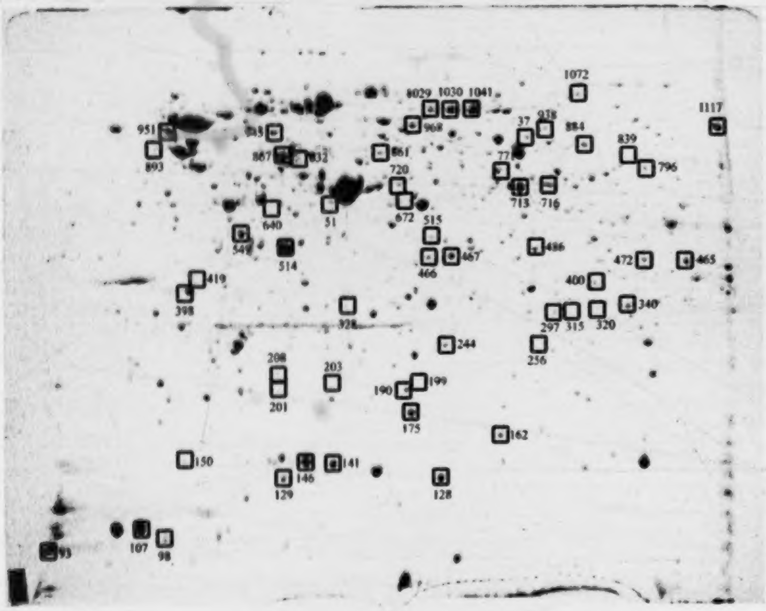
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Figure 4.1. Protein expression profile of cold adapted, log phase *E. coli* after 1 day of incubation at 15°C. Differentially expressed protein spot (☐)

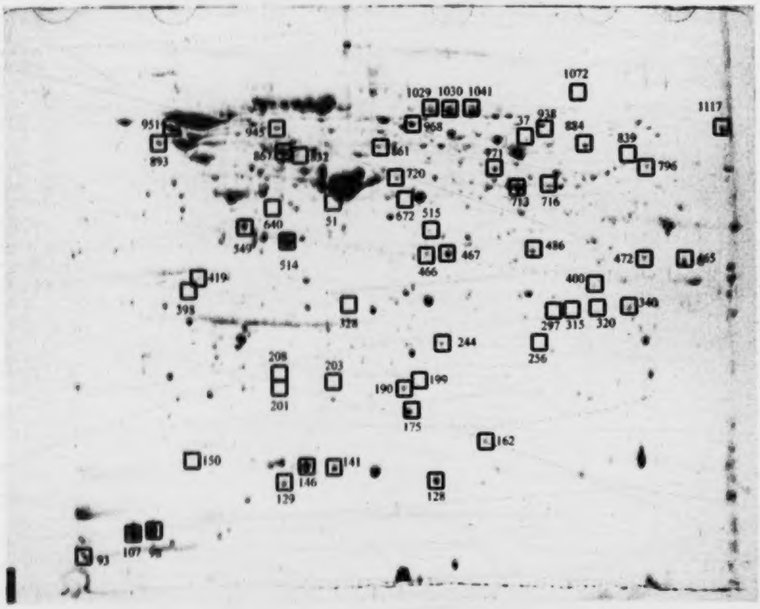
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Figure 4.2. Protein expression profile of cold adapted, log phase E. coli after 1 day of incubation at 2°C. Differentially expressed protein spot (☐)

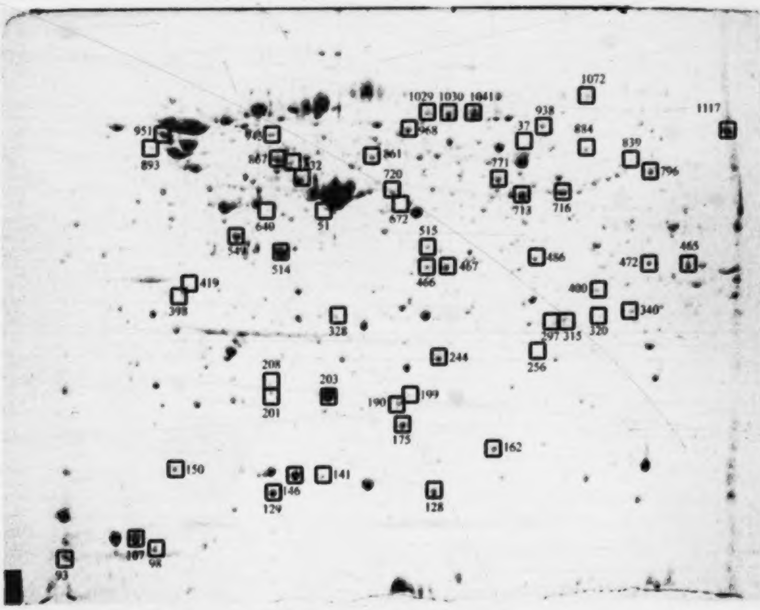
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Figure 4.3. Protein expression profile of cold adapted, log phase *E. coli* after 1 day of incubation at 6°C. Differentially expressed protein spot (☐)

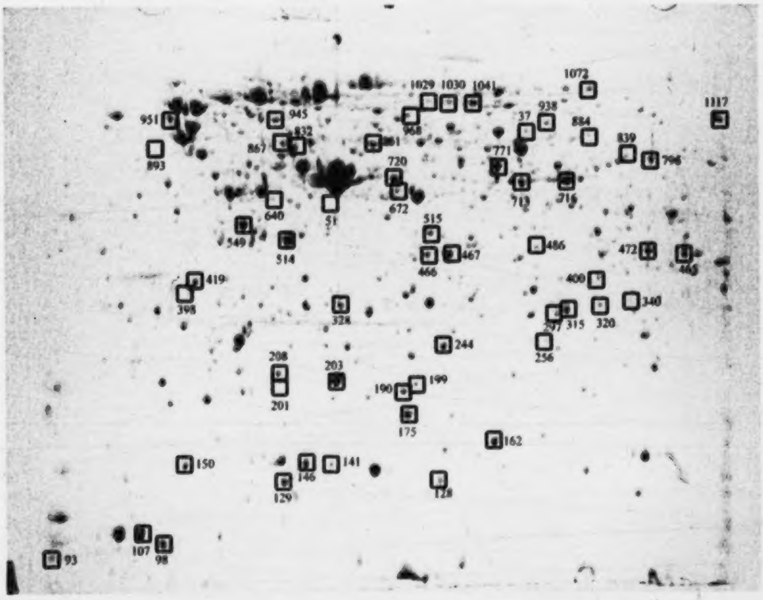
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Figure 4.4. Protein expression profile of cold adapted, log phase *E. coli* after 4 days of incubation at 6°C. Differentially expressed protein spot (☐)

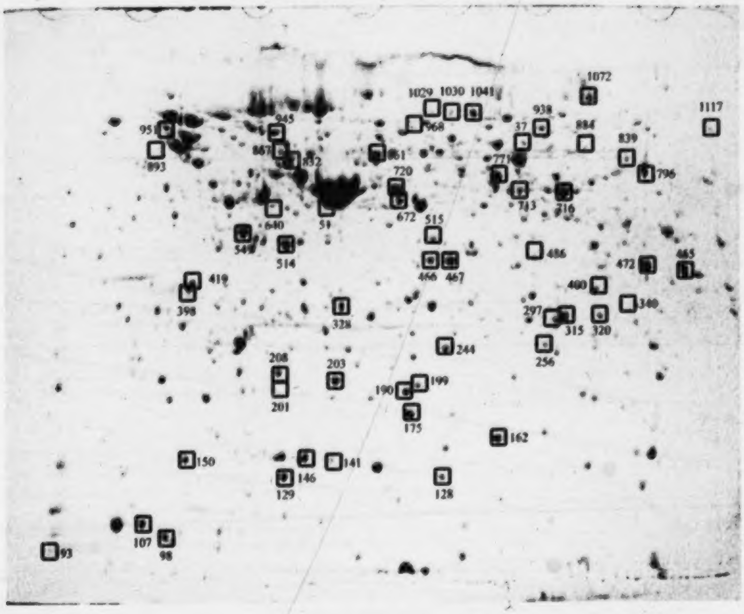
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Figure 4.5. Protein expression profile of cold adapted, log phase *E. coli* after 8 days of incubation at 6°C.

of the spots contained a single protein while spot 93 contained 2 ribosomal subunit proteins (Table 4.2). However, spots 117, 299, 568, 775, 798, and 1002 contained multiple unrelated proteins of similar masses, and were not included in further analyses. Ribosomal proteins were among the proteins detected in spots 117 and 1002. Several proteins were represented by more than one differentially expressed protein spot; spots 201 and 203 were identified as osmotically inducible periplasmic protein (OsmY), spots 141 and 146 were identified as ribosomal subunit protein S6, and spots 1029, 1030, and 1041 were identified as pyruvate formate acetyltransferase 1. Ribosomal proteins were among the most abundant proteins detected at 15°C that were differentially expressed. Ribosomal subunit proteins S15/S18, S6, and L7/L15 and elongation factor EF-Tu/EF-Ts together made up about 4% of the total amount of protein expressed at 15°C; while dihydropteridine reductase, pyridine nucleotide transhydrogenase, and OsmY were among the least abundant proteins at 15°C that were differentially expressed.

Expression of 38 proteins was greater after 8 days of incubation at 6°C relative to levels expressed at 15°C (Table 4.3). Of those 38 proteins, 27 had increased levels after 1

Table 4.2. Differential expression and identification of proteins expressed by cold adapted, log phase *E. coli* after incubation at 2 or 6°C.

Spot ID	% of total protein at 15°C	Mass	pI	Protein ID
37	0.018	61099	5.95	Oligopeptide transport; periplasmic binding protein
51[a]	0.098	41652	5.37	Succinyl-CoA synthetase, β subunit
93	0.788	9994	10.40	30S ribosomal subunit protein S15
93	0.788	9038	10.60	30S ribosomal subunit protein S18
98[a]	0.122	12000	4.67	Thioredoxin
107	0.940	12288	4.60	50S ribosomal subunit protein L7/L12
128	0.323	15511	5.54	Nucleoside diphosphate kinase
129	0.168	10381	5.15	GroES
141	0.364	15177	5.26	30S ribosomal subunit protein S6
146	0.706	15177	5.26	30S ribosomal subunit protein S6
150	0.042	14011	4.82	IscU-NifU homologue
162	0.139	17012	5.68	Ferric uptake regulator
175	0.287	18270	5.52	Peptidyl-prolyl cis-trans isomerase B
190	0.116	18993	5.44	Single strand DNA binding protein
199	0.019	22540	6.28	Outer-membrane lipoprotein carrier protein precursor
201	absent	21061	6.32	Osmotically inducible periplasmic protein (OsmY)
203	0.010	21061	6.32	Osmotically inducible periplasmic protein (OsmY)
208	0.033	19603	5.18	S-ribosylhomocysteinase
244	0.088	21663	5.55	ClpP protease, chain A
256[a]	0.015	21877	5.84	Modulator of drug activity B, significant alignment with flavodoxin, flavodoxin like fold
297	0.039	23673	5.86	Disulfide Bond Isomerase (DsbC), chain A
315	0.035	27357	6.37	Hypothetical oxidoreductase
320	0.016	23473	6.06	Glucose inhibited division protein B (GidB)
328	0.023	25477	5.39	Phage shock protein A
340[a]	0.112	27732	6.07	Fumarate reductase, iron-sulfur protein subunit
398	0.448	21182	4.86	FKBP-type peptidyl-prolyl cis-trans isomerase
400	0.047	28528	6.00	Pyruvate formate lyase activating enzyme 1
419	0.063	27671	4.84	Probable ATP-dependent transporter
465	0.159	29168	6.12	Extragenic gene suppressor (SuhB)
466	0.088	25524	5.54	RNase PH
467	0.381	28074	5.58	Enoyl-acyl-carrier-protein (ACP) reductase, NADH dependent
472	0.078	29913	6.31	Succinyl-CoA synthetase, α subunit
486[a]	0.180	33735	5.89	orf, conserved hypothetical protein-significantly related to mutarotase (aldose epimerase)
514	1.202	30387	5.22	Elongation Factor Complex EF-Tu EF-Ts, chain B
515[a]	0.051	32488	5.61	Malate dehydrogenase
549	0.316	35237	5.10	Transaldolase B, chain A
640	0.017	32866	5.07	Quinolinate phosphoribosyltransferase
672	0.003	43924	5.48	Dihydropteridine reductase
713	0.600	43605	5.85	Acetate kinase
716	0.479	42206	5.94	Cysteine desulfurase
720	0.092	46498	5.49	Peptidase B

771	0.239	51652	5.68	Probable adenylosuccinate lyase
796	0.050	48444	6.32	Citrate synthase
832[a]	0.136	49452	5.18	L-serine deaminase
839	0.009	49879	6.28	Pyridine nucleotide transhydrogenase, soluble
861[a]	0.114	43984	5.58	2-oxoketoglutarate dehydrogenase, dihydrolipoamide succinyltransferase component
867[a]	0.693	54714	5.54	Aspartate ammonia-lyase
884	0.131	54891	5.91	IMP dehydrogenase subunit
893	0.036	48058	4.72	Flagellin
938	0.017	62563	5.82	Pyruvate oxidase
945[a]	0.235	57638	5.11	Lysine-tRNA synthetase
951	0.642	63722	4.78	PEP-protein phosphotransferase system enzyme I
968	0.231	67095	5.56	Glucosamine-6-P-synthetase
1029[a]	0.278	85588	5.69	Pyruvate formate lyase I
1030[a]	0.509	85588	5.69	Pyruvate formate lyase I
1041[a]	0.844	85588	5.69	Pyruvate formate lyase I
1072[a]	0.098	89390	6.23	Lon protease
1117	0.433	70560	8.75	Cold-shock DEAD-box protein A (ATP-dependent RNA helicase deaD)

[a] Differential expression detected only in one set of gels

day of incubation at 6°C while 6 had decreased levels at that time. The most dramatic increase in differential expression was observed for OsmY at spot 203, expression of which had increased more than 100-fold after 1 day of incubation at 6°C. Expression of OsmY decreased to levels that after incubation for 8 days at 6°C were about 30 times higher than that found at 15°C (Fig. 4.6). OsmY at spot 201 was not detected at 15°C, after 1 day of incubation at 2°C or after 8 days of incubation at 6°C, but represented 0.06 or 0.03 % of the total protein expressed after 1 or 4 days of incubation at 6°C, respectively (data not shown). Expression of dihydropterine reductase had increased almost 100-fold after 8 days of incubation at 6°C (Fig. 4.7). While most proteins were moderately induced, 10-fold increases after 8 days of incubation at 6°C were observed for both α and β subunits of succinyl-CoA synthetase, the gene product of *yjgI*, and phage shock protein A (PspA).

A number of the upregulated proteins are involved in the cellular functions of protein folding, protein degradation, protein transport, carbohydrate metabolism and electron transport, and in the tricarboxylic acid (TCA) cycle. Ferric uptake regulator (Fur), PspA, S-ribosylhomocysteinase, and glucose inhibited division protein B (GidB; Fig. 4.8) are proteins involved in various regulatory pathways that were all increasingly upregulated with increasing incubation time at 6°C.

Table 4.3 Identification of proteins, their gene, function and relative expression levels at 6 and 2°C for proteins that display increased expression in cold adapted, log phase *E. coli* after 8 days of incubation at 6°C relative to levels expressed at 15°C.

Protein	**Gene**	**General Function**	**Differential Expression**			
			6°C (1 d)	**6°C (4 d)**	**6°C (8 d)**	**2°C (1 d)**
RNase PH	*rph*	tRNA processing, mRNA degradation	1.2	1.7	2.5	1.0
Lysine-tRNA synthetase[a]	*lysS*	protein synthesis	0.7	0.9	1.5	0.7
L-serine deaminase[a]	*sdaA*	amino acid degradation	1.2	2.4	2.2	0.6
Peptidyl-prolyl cis-trans isomerase B	*ppiB*	protein folding	1.0	1.4	1.5	1.0
GroES	*groS*	protein folding	1.9	1.7	1.8	1.0
Disulphide Bond Isomerase (DsbC), chain A	*dsbC*	protein folding	1.4	4.4	4.0	0.6
ClpP protease, chain A	*clpP*	protein degradation	2.5	2.5	2.5	1.0
Lon protease[a]	*lon*	protein degradation	1.4	1.4	3.0	0.4
Peptidase B	*pepB*	protein degradation	1.9	6.9	5.7	0.8
Oligopeptide transport; periplasmic binding protein	*oppA*	protein transport	0.5	4.3	4.1	0.5
Outer-membrane lipoprotein carrier protein precursor	*lolA (lplA)*	protein transport	1.9	4.2	5.2	0.6
Probable adenylosuccinate lyase	*purB*	nucleotide biosynthesis	1.3	3.2	3.8	0.9
Enoyl-ACP reductase, NADH dependent	*fabI*	fatty acid biosynthesis	0.5	0.9	1.8	1.0
Citrate synthase	*gltA*	TCA cycle	3.3	4.9	5.9	1.0
Succinyl-CoA synthetase, α subunit	*sucC*	TCA cycle	1.4	7.4	10.3	1.0
Succinyl-CoA synthetase, β subunit[a]	*sucC*	TCA cycle	2.3	0.0	9.4	1.4
2-oxoketoglutarate dehydrogenase, dihydrolipoamide succinyltransferase component[a]	*sucB*	TCA cycle	1.4	2.7	5.2	0.9
Malate dehydrogenase[a]	*mdh*	TCA cycle	1.7	3.4	3.8	1.0
Transaldolase B, chain A	*talB*	carbohydrate metabolism	1.2	1.7	1.8	1.1
Pyruvate formate lyase activating enzyme 1	*pflA (act)*	carbohydrate metabolism, anaerobic	0.8	1.4	1.9	0.7
Extragenic suppressor	*suhB (ssyA)*	carbohydrate transport and metabolism, regulator(?)	1.0	2.3	3.1	1.1
Pyruvate oxidase	*poxB*	electron transport	4.1	5.4	5.4	0.0
Pyridine nucleotide transhydrogenase, soluble	*sthA (udhA)*	electron transport	1.6	3.6	5.7	1.2

Table 4.3 continued

Protein	*Gene*	General Function	Differential Expression			
			6°C (1 d)	6°C (4 d)	6°C (8 d)	2°C (1 d)
Hypothetical oxidoreductase	*yjgI*	electron transport (hypothetical)	2.4	10.0	10.0	0.5
Dihydropteridine reductase	*hmp (hmpA, fsrB)*	electron transport, Fe scavenging	6.1	31.3	94.2	0.0
Modulator of drug activity B, significant alignment with Flavodoxin, flavodoxin like fold[a]	*mdaB (mda66)*	electron transport	1.0	2.7	4.8	0.9
Thioredoxin[a]	*trxA (tsnC, fipA)*	electron transport	1.1	2.4	2.2	0.9
Probable ATP-dependent transporter	*sufC*	ABC transport family (probable)	0.6	3.0	3.2	0.9
IscU-NifU homologue	*iscU (nifU)*	Fe-S cluster assembly (probable)	2.3	4.3	4.8	0.8
Cysteine desulfurase	*iscS*	Fe-S cluster assembly, NAD biosynthesis	0.8	1.4	1.6	0.3
Quinolinate phosphoribosyltransferase	*nadC*	NAD biosynthesis	1.2	2.8	3.6	0.9
Ferric uptake regulator	*fur*	ferric iron uptake, regulator	1.3	2.3	2.5	0.9
S-ribosylhomocysteinase	*luxS*	autoinducer-2 synthesis, cell-cell signalling	1.3	4.4	5.5	0.5
Phage shock protein A	*pspA*	regulator	3.0	9.3	10.9	0.7
Osmotically inducible periplasmic protein (OsmY)	*osmY*	stress protein	122.4	83.0	33.7	0.8
Glucose inhibited division protein B (GidB)	*gidB*	cell division, DNA replication	1.3	3.9	5.4	1.0
Single-strand DNA-binding protein	*ssb (exrB, lexC)*	DNA replication	1.1	1.7	2.2	1.1

[a] Differential expression detected only in one set of gels

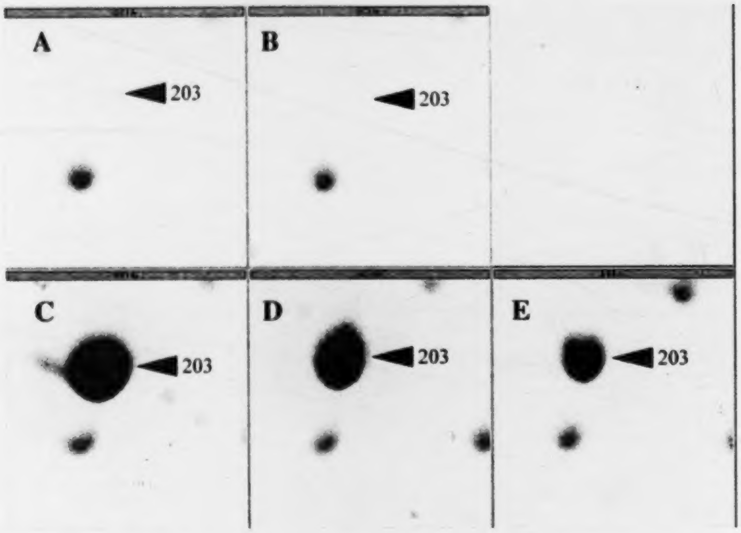
A
203
B
203
C
203
D
203
E
203

Figure 4.6. Expression of OsmY at spot 203 after incubation of *E. coli* at 15°C for 1 day (A) followed by incubation at 2°C for 1 day (B) or 6°C for 1 (C), 4 (D) or 8 (E) days.

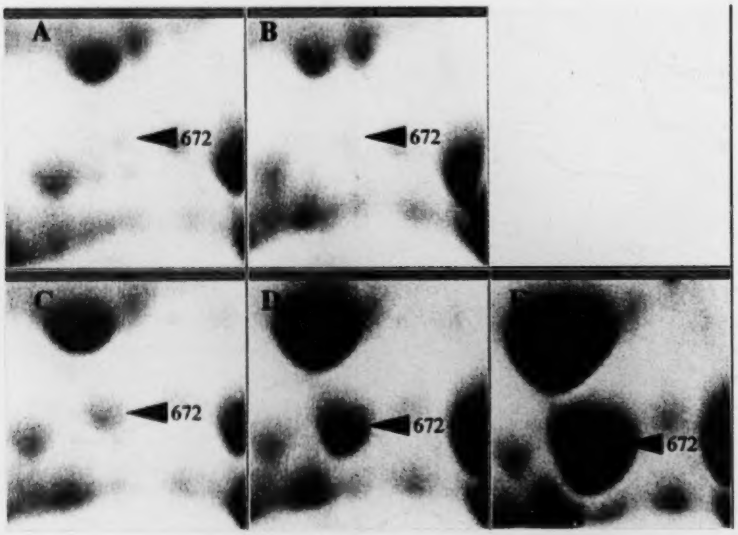
A
672
B
672
C
672
D
672
672

Figure 4.7. Expression of dihydropteridine reductase at spot 672 after incubation of *E. coli* at 15°C for 1 day (A) followed by incubation at 2°C for 1 day (B) or 6°C for 1 (C), 4 (D) or 8 (E) days.

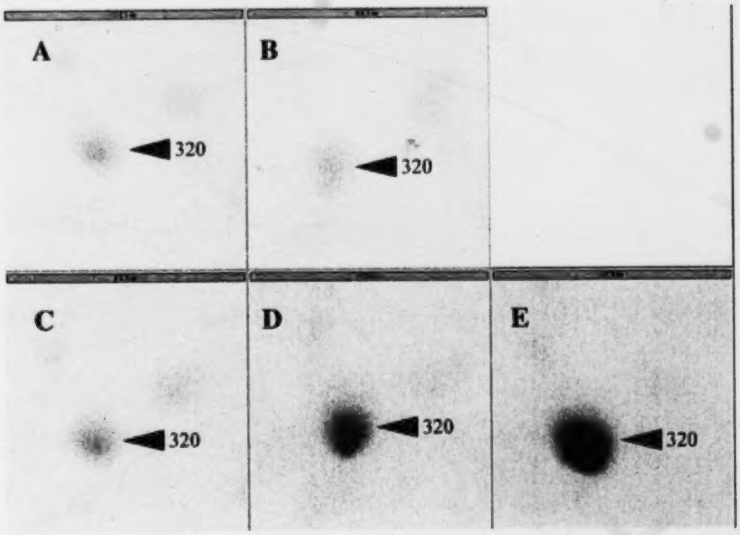

Figure 4.8. Expression of GidB at spot 320 after incubation of *E. coli* at 15°C for 1 day (A) followed by incubation at 2°C for 1 day (B) or 6°C for 1 (C), 4 (D) or 8 (E) days.

Most of the proteins that had increased expression after 8 days of incubation at 6°C had a slight decrease in expression after 1 day of incubation at 2°C compared with the levels expressed at 15°C. Pyruvate oxidase and dihydropteridine reductase were expressed at low levels at 15°C (Table 4.2) and were not detected after 1 day of incubation at 2°C, but the levels of both proteins increased after 1 day of incubation at 6°C (Table 4.3).

Relative to levels expressed at 15°C, expression of 19 proteins decreased after 8 days of incubation at 6°C (Table 4.4). Levels of 18 of the 19 proteins decreased or remained unchanged after 1 day of incubation at 6°C, while levels of the cold-shock DEAD-box protein A (CsdA) increased after 1 day of incubation at 6°C and then decreased after 8 days of incubation at 6°C to levels that were about 10-fold lower than levels expressed at 15°C, (Fig. 4.9). A 20-fold reduction was observed for fumarate reductase after 8 days of incubation at 6°C. This was the largest decrease in relative expression.

Table 4.4 Identification of proteins, their gene, function and relative expression levels at 6 and 2°C for proteins that display decreased expression in cold adapted, log phase *E. coli* after 8 days of incubation at 6°C relative to levels expressed at 15°C.

Protein	**Gene**	**General Function**	**Differential Expression**			
			6°C (1 d)	**6°C (4 d)**	**6°C (8 d)**	**2°C (1 d)**
30S ribosomal subunit protein S15/S18	*rpsO, rpsR*	protein synthesis	0.3	0.2	0.2	0.5
30S ribosomal subunit protein S6	*rpsF*	protein synthesis	0.2	0.2	0.2	0.6
30S ribosomal subunit protein S6	*rpsF*	protein synthesis	0.8	0.6	0.4	1.1
50S ribosomal subunit protein L7/L12 (L8)	*rplL*	protein synthesis	0.8	0.6	0.3	1.1
Elongation Factor Complex EF-Tu/EF-Ts, chain B	*tufA, tsf*	protein synthesis	0.8	0.6	0.4	1.5
Cold-shock DEAD-box protein A (ATP-dependent RNA helicase deaD)	*csdA (deaD)*	cold shock ribosomal factor (RNA unwinding)	1.7	0.6	0.1	1.6
FKBP-type peptidyl-prolyl cis-trans isomerase	*slyD*	protein folding	0.2	0.3	0.3	0.2
IMP dehydrogenase subunit	*guaB*	nucleotide biosynthesis, energy metabolism	0.6	0.3	0.2	0.8
Nucleoside diphosphate kinase [a]	*ndk*	nucleotide biosynthesis	0.6	0.5	0.4	1.0
Acetate kinase	*ackA*	energy/carbon metabolism, anaerobic	0.9	0.6	0.5	1.0
Fumarate reductase, iron-sulfur protein subunit [a]	*frdB*	energy/carbon metabolism, anaerobic	0.6	0.2	0.05	0.8
Pyruvate formate lyase I, induced anaerobically [a]	*pflB*	energy/carbon metabolism, anaerobic	0.5	0.2	0.0	0.8
Pyruvate formate lyase I, induced anaerobically [a]	*pflB*	energy/carbon metabolism, anaerobic	0.6	0.3	0.2	0.9
Pyruvate formate lyase I, induced anaerobically [a]	*pflB*	energy/carbon metabolism, anaerobic	0.8	0.4	0.3	0.7
Aspartate ammonia-lyase [a]	*aspA*	carbon metabolism, anaerobic	0.7	0.2	0.2	0.9
Glucosamine-6-P-synthetase	*glmS*	glucosamine metabolism	1.0	0.4	0.2	1.0
orf, conserved hypothetical protein- significantly related to mutarotase (aldose epimerase) [a]	*yeaD*	hexose metabolism (hypothetical)	0.5	0.3	0.1	0.9
PEP-protein phosphotransferase system enzyme I	*ptsI*	carbohydrate transport	0.5	0.3	0.4	0.6
Flagellin	*fliC*	flagella	0.9	0.2	0.2	4.0

[a] Differential expression detected only in one set of gels

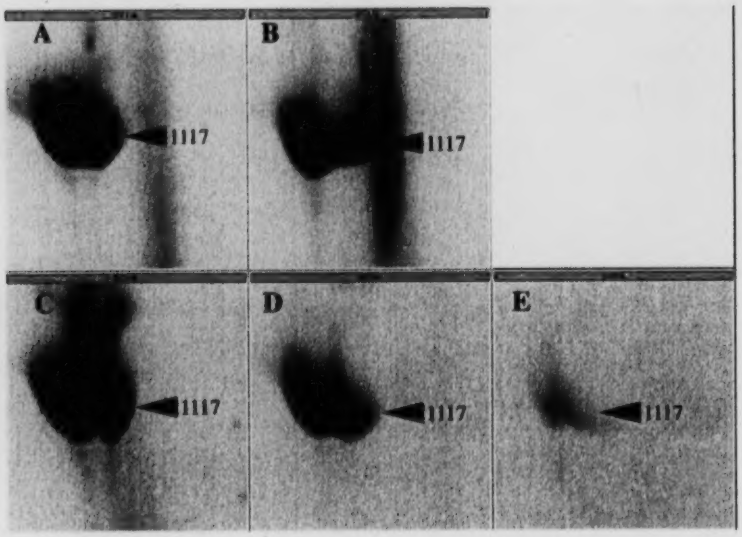

Figure 4.9. Expression of CsdA at spot 1117 after incubation of *E. coli* at 15°C for 1 day (A) followed by incubation at 2°C for 1 day (B) or 6°C for 1 (C), 4 (D) or 8 (E) days.

A number of the downregulated proteins are involved in protein synthesis, nucleotide biosynthesis or anaerobic carbon metabolism and energy generation. While levels of most proteins that were downregulated after 8 days of incubation at 6°C did not change substantially or decreased after 1 day at 2°C, levels of flagellin (FliC) increased 4-fold after 1 day at 2°C, and were unchanged after 1 day of incubation at 6°C, but had decreased 5-fold after 8 days at 6°C (Fig. 4.10). In addition, levels of CsdA and elongation factor EF-Tu/EF-Ts were slightly elevated after 1 day at 2°C relative to the levels expressed at 15°C.

4.4 Discussion

4.4.1 Behaviour of cold adapted, log phase E. coli *at 2 and 6°C*

Previous studies on the behaviour of cold adapted, log phase *E. coli* at temperatures below the minimum for sustained growth indicated that growth ceased at 2°C but not at temperatures > 2°C (Gill et al., 2001; Chapter 2). After 1 day of incubation, absorbance values did not increase for cultures incubated at 2°C but increased for cultures incubated at temperatures >2°C. At 6°C, viable cell numbers increased after

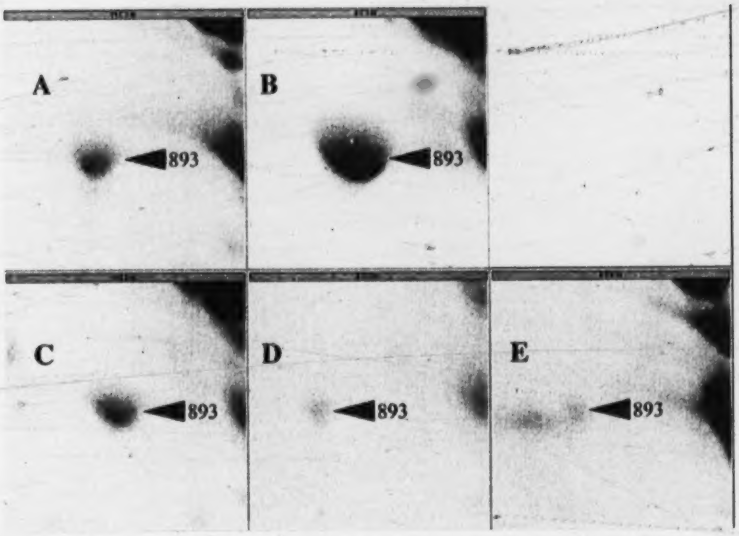

Figure 4.10. Expression of flagellin at spot 893 after incubation of *E. coli* at 15°C for 1 day (A) followed by incubation at 2°C for 1 day (B) or 6°C for 1 (C), 4 (D) or 8 (E) days.

1 day of incubation but declined with further incubation, although absorbance values continued to increase while a fraction of the cells elongated to form filaments after 5 days of incubation. Based on those findings, the proteomic response of cold adapted, log phase *E. coli* cultures was examined after 1 day of incubation at 2°C and after 1, 4 and 8 days of incubation at 6°C. This was done to compare the effect of absence of growth to growth after 1 day of incubation at temperatures below the minimum for sustained growth, and to examine the effects of longer incubation until cell elongation becomes apparent. The cold adapted, log phase culture prepared at 15°C was used as the reference culture. Results of the current study agree with findings of previous studies (Chapters 2 and 3) that growth ceased after 1 day of incubation at 2°C but not at 6°C and that cell elongation was not apparent after 4 days but was observed after 8 days of incubation at 6°C.

4.4.2 Two-dimensional gel electrophoresis

Analysis of 2-D gels of the proteins expressed by cold adapted, log phase *E. coli* before and after incubation at temperatures below the minimum for sustained growth by image analysis software identified differential expression of 63 distinct protein spots that were subsequently analyzed by mass spectrometry. Of the 63 spots, 53 diverse proteins were represented by spots that contained a single protein although some proteins were located in different regions of the gel than anticipated. For example, the theoretical pI of spot 93 is above 10 but the spot was located on the acidic edge of the gel. Spots 201 and 203 were identified as OsmY, which has a theoretical pI of 6.3, but both were detected in the middle of the pI range on the gel. As proteins were equally distributed along the IEF strip during rehydration and migrate according to their charges the discrepancies in location were not related to IEF, but could be a result of modification of proteins that may occur naturally in the cell or during sample preparation. Discrepancies between theoretical pI values and observed location on gel images have been observed for other *E. coli* proteins (Mihoub et al., 2003).

4.4.3 Proteomic analysis of cold adapted, log phase E. coli *cultures at 2°C*

Previous studies showed absorbance values did not increase in cold adapted, log phase *E. coli* cultures during incubation at 2°C, which suggests that growth had ceased at that temperature (Gill et al., 2001; Chapters 2 and 3). Indeed, levels of most proteins that were differentially expressed at 6°C did not change substantially or decreased after 1 day at 2°C, with the exception of CsdA and elongation factor EF-Tu/EF-Ts, which were slightly elevated after 1 day at 2°C and FliC, which increased 4-fold after 1 day at 2°C relative to the levels expressed at 15°C. CsdA and EF-Tu/EF-Ts are induced during the cold shock response (Jones et al., 1987; 1996), therefore, it is possible that levels of CsdA and EF-Tu/EF-Ts may have increased during the early hours of incubation at 2°C. The reason for increased levels of FliC at 2°C is not clear; however, flagella biosynthesis is a highly regulated process (Macnab, 1996). Perhaps the increase in levels of FliC at 2°C may be related to a regulatory effect, although the mechanism is not known.

4.4.4 Production of cold shock proteins in cold adapted, log phase E. coli *at 6°C*

Shifts of temperature within the normal growth range generally result in quick, transient adjustments of the level of cell components and enzyme activity in log phase cells, but when cells are shifted to temperatures above or below the normal growth range, major changes are necessary for growth to continue. Adaptation and growth of mesophilic cells at cold temperatures requires that the structural integrity of membranes, proteins and ribosomes is maintained and the ability to carry out protein synthesis and nutrient uptake is preserved (Berry and Foegeding, 1997). However, a single determinant for adaptation to low temperatures has not been identified (Berry and Foegeding, 1997). In addition, there is a lack of information on the behaviour of mesophilic pathogens such as *Salmonella* and *E. coli* at chiller temperatures because they are not generally expected to grow at temperatures about 7°C (Matches and Liston, 1968; Shaw et al., 1971). Moreover, studies of the response to cold of *E. coli* have concentrated on the initial, transient cold shock response to cold temperatures while the expression of cold acclimation proteins during continuous growth at low temperatures has not been studied (Phadtare et al., 1999).

It is well established that a block in initiation of translation is the major factor that limits the growth of mesophiles at low temperatures (Jones and Inouye, 1994). Even during growth at 15°C, translation does not occur at an optimal rate as the fraction of nontranslating ribosomes in cells is three times higher than that found during growth at 37°C (Farewell and Neidhardt, 1998). Cold shock protein A (CspA) may act like a RNA chaperone by binding with broad specificity to mRNA to prevent secondary folding at low temperatures, while cold shock ribosomal factors CsdA and RbfA are ribosomal components that are essential at low temperatures for translation of non-cold shock mRNAs (Jiang et al., 1997; Jones et al., 1996; Jones and Inouye, 1996). CspA, RbfA, and CsdA are produced at basal levels at low temperatures. In the current study, changes in levels of expression of CspA and RbfA were not detected when cold adapted, log phase *E. coli* cultures were incubated at 6°C but CsdA levels did increase after 1 day of incubation at 6°C. However, levels decreased about 10-fold after 8 days of incubation, relative to levels expressed at 15°C. Such a decrease would not be expected to occur if growth was sustained at 6°C. Therefore, these findings suggest that translation is not sustained with increased incubation time at 6°C.

4.4.5 General stress response

Most bacteria are able to adapt to and grow under a range of changing environmental conditions. However, when environmental conditions become extreme for sustained growth, cells must preserve the integrity of the DNA and maintain an intact cell membrane and proper folding of proteins to maximize their chances for survival (Booth, 2002). To avoid a random arrest of the cell cycle when encountering growth limiting conditions, cell components such as DNA, protein, and cell mass no longer increase at the same rate to enable cells to enter the stationary phase in an orderly fashion (Kolter et al., 1993). *E. coli* cells become smaller and develop a spherical instead of a rod-shaped morphology upon exposure to sudden environmental stresses and during the transition to the stationary phase (Aldea et al., 1989; Santos et al., 1999). The round morphology is dependent on active FtsZ and is associated with overexpression of *bolA* (Aldea et al., 1988). Transcription of *bolA* is constitutive from promoter P2 and inducible from the main promoter P1 (Aldea et al., 1988; 1989; Lange and Hengge-Aronis, 1991). Growth rate and growth phase dependent expression of *bolA* from promoter P1 is dependent on the starvation or stationary phase sigma factor σ^S (RpoS), the product of the *rpoS* gene, while expression from P1 when cells are exposed to environmental stress is dependent or independent of RpoS (Aldea et al., 1989; Lange and Hengge-Aronis, 1991; Loewen et al., 1998; Santos et al., 1999; Tanaka et al., 1993).

Determinations of cell lengths have consistently shown that lengths of cells decreased and the fraction of cells that gave FALS measurements below the 90th percentile reference value increased after 1 day of incubation at 6°C (Chapters 2 and 3 and Table 4.1), which indicates that cold adapted, log phase *E. coli* become smaller after 1 day of incubation at 6°C. Furthermore, levels of OsmY increased > 100-fold after 1 day of incubation at 6°C. The *osmY* gene encodes a periplasmic protein of unknown function but the *osmY* promoter can be used as an indicator of RpoS levels (Colland et al., 2000; Hengge-Aronis et al., 1993; Yeats and Bateman, 2003; Yim and Villarejo, 1992; Yim et al., 1994). The findings of the current study suggest that RpoS levels dramatically increased after 1 day of incubation at 6°C and then declined with increasing incubation time. However, levels of OsmY were still about 30 times higher after 8 days of incubation at 6°C than was found in cells incubated at 15°C. A decrease in the stability of *osmY* mRNA, due to increased mRNA degradation or secondary structures would lead to a decrease in levels of OsmY. As mRNA levels were not determined in this study, there may not be a direct relationship between levels of RpoS and OsmY during incubation at 6°C after the initial increase in OsmY levels.

The cellular level of RpoS is controlled by a series of complex mechanisms that affects transcription, translation, and post-translational stability, with different stress conditions differentially affecting the various levels of control (Hengge-Aronis, 2000). A gradual deterioration of environmental conditions appears to stimulate *rpoS* transcription or translation while potentially lethal conditions seem to affect RpoS proteolysis (Hengge-Aronis, 2000). The transcription of *rpoS* is inversely correlated with growth rate and positively correlated with levels of the alarmone nucleosides guanosine tetraphosphate (ppGpp) and pentaphosphate (pppGpp) (Lange and Hengge-Aronis, 1994; Lange et al., 1995).

Levels of (p)ppGpp decrease during a temperature downshift (Jones et al., 1992). However, when *E. coli* cells experience conditions of limiting amounts of available energy, carbon source or amino acids, a stringent response is invoked. This response involves the accumulation of the alarmone (p)ppGpp, which triggers changes that allow for long term survival and general resistance to various other environmental stresses, including unfavourable temperatures, pH, osmolarity and exposure to reactive oxygen and nitrogen species (Moat et al., 2002c). The initial response to carbon-energy source limitation is the appearance or increased expression of high affinity nutrient utilization systems, with scavenging of carbon and energy sources and increased metabolic efficiency. These metabolic changes are mediated by the accumulation of (p)ppGpp and are regulated by RpoS and additional global regulators (Moat et al., 2002c). Other physiological changes that occur during the stringent response include the degradation of cellular RNA, proteins, and fatty acids, alteration of the lipid composition of the cell membrane and the condensation of chromosomal DNA, which protects it from damage (Cashel et al., 1996).

The majority of proteins that were upregulated after 1 day of incubation at 6°C are involved in carbon metabolism, the TCA cycle and electron transport, which are all associated with processes that provide energy; or with protein folding and degradation, which are processes that place additional energy demands on cellular resources. The majority of the downregulated proteins are involved in protein or nucleotide biosynthesis, or anaerobic carbon or energy metabolism, which would seem to be unusual under aerobic conditions. The rate of chemical reactions is inversely related to temperature; thus cells have an unavoidable increased demand for energy at low temperatures as higher activation energy for enzymatic reactions is required. Therefore, the changes in the proteome of *E. coli* at 6°C suggest that cells are struggling to generate the amount of energy required to maintain growth. Cells that cannot sustain growth will gradually die as the demand for maintenance energy exceeds the rate of generation (Ingram and Mackey, 1976). The following analysis of the proteomic response of cells at temperatures just below the minimum for growth provides insight into the behaviour of cells at such temperatures, which may be extended to possibly explain similar behaviours observed during other stresses.

4.4.6 Induction of the stringent response

During growth of *E. coli* at 15°C, there is an excess of nontranslating ribosomes (Farewell and Neidhardt, 1998). Because the rate of peptide elongation per ribosome is constant, fewer ribosomes are required as the growth rate decreases (Moat et al., 2002a). Indeed, decreases in ribosomal subunits and elongation factor EF-Tu/EF-Ts were observed after 1 day of incubation at 6°C. These findings are consistent with well known facts that levels of ribosomal proteins are subject to translational repression and initiation of translation is inhibited at temperatures below the minimum for growth (Jones and Inouye, 1994; Moat et al., 2002a). However, levels of ribosomal proteins continued to decrease with increasing incubation time at 6°C. A considerable amount of energy is required for ribosome synthesis, thus when *E. coli* experiences conditions of limiting amounts of available energy, cells conserve energy by invoking the stringent response (Cashel et al., 1996).

Accumulation of (p)ppGpp results in a decrease in transcription of genes encoding ribosome associated components such as rRNA and tRNA. That is supposed to be the result of (p)ppGpp binding to the β-subunit of RNA polymerase, which could reduce the stability of the open promoter/RNA polymerase complexes at rRNA promoters (Chatterji et al., 1998; Kvint et al., 2000). In addition, CspA is negatively regulated by (p)ppGpp (Jones et al., 1992). Therefore, as levels of (p)ppGpp increase during the stringent response, fewer CspA molecules would be available for efficient mRNA translation, which may further contribute to decreased protein synthesis at 6°C.

SuhB may act as an RNA chaperone by binding to mRNA to prevent secondary folding at low temperatures, which prevents attack from ribonuclease III (Chen and Roberts, 2000; Inada and Nakamura, 1995; 1996). Levels of SuhB increased 2- fold after 4 days of incubation at 6°C and were 3-fold higher after 8 days relative to levels at 15°C, which suggest that SuhB could preserve mRNA for translation when conditions become favourable for growth.

As the stringent response progresses past the initial stages, severe inhibition of pppGpp synthesis probably occurs. However, the cause of this inhibition is not known (Cashel et al., 1996). Conversion of GDP to GTP, catalyzed by nucleoside diphosphate kinase (Ndk), regenerates GTP for pppGpp synthesis (Cashel et al., 1996). However, levels of Ndk decreased with increasing incubation time at 6°C, which suggests that less GDP is being converted to GTP at 6°C than at 15°C, which may lead to cells becoming depleted of GTP. FtsZ has GTPase activity and the assembly of the FtsZ ring is dependent on GTP while disassembly of the FtsZ filaments occurs when GTP is depleted (Bramhill and Thompson, 1994; Yu and Margolin, 1997). Because cells contain between 10,000 to 20,000 molecules of FtsZ, there presumably is a great demand for GTP (Erickson et al., 1996; Rothfield and Justice, 1997). During the stringent response, cells consume ATP to convert GTP into pppGpp, which may deplete cells of GTP and the energy in general that is required for cell division. Therefore, it is plausible that cell division is simply inhibited due to decreased levels of GTP, or perhaps is due to some other indirect effect of (p)ppGpp.

4.4.7 Quorum sensing signals

Lysine-tRNA synthetase is unusual because it is encoded by two distinct genes, *lysS* and *lysU*, while other aminoacyl-tRNA synthetases are encoded by a single gene (Hirschfield et al., 1981; 1984). While demand for aminoacyl-tRNA synthetases and amino acids in general is reduced under growth restrictive conditions, lysine-tRNA synthetase encoded by *lysS* is expressed constitutively while expression from *lysU* is induced by various environmental stimuli (Grunberg-Manago, 1996; Neidhardt and VanBogelen, 1981). In the current study, levels of LysS decreased slightly after 1 day of incubation at 6°C and increased slightly with increasing incubation time, which may be expected because lysine and threonine biosynthetic enzymes are induced during the stringent response due to imbalances in amino acid biosynthetic pathways as carbon and energy sources are depleted (Cashel et al., 1996; Grunberg-Manago, 1996). Higher threonine levels lead to feedback inhibition and accumulation of intermediates of the threonine biosynthetic pathway such as homoserine and homoserine phosphate, which may lead to biosynthesis of homoserine lactone (Huisman and Kolter, 1994). Homoserine lactone may be an intracellular signal that accumulates in cells and possibly induces transcription of *rpoS* (Huisman and Kolter, 1994).

Many species of bacteria modulate gene expression in response to a cell to cell signalling process known as quorum sensing. Many gram negative bacteria produce acylated homoserine lactones [(AHLs or autoinducer-1 (AI-1)] or autoinducer-2 (AI-2), which is synthesized from adenosylmethionine, as signalling molecules (Fuqua et al., 2001; Huisman and Kolter, 1994; Miller et al., 2004; Surette et al., 1999). Microorganisms initiate a signal transduction cascade when a threshold stimulatory concentration of AHL is reached in the local environment as a result of increased population density or possibly stress (Fuqua et al., 2001; Huisman and Kolter, 1994). Production and degradation of AI-2 is influenced by the metabolic potential of the environment where maximal secretion of AI-2 is observed in the logarithmic phase of growth while AI-2 is degraded during the stationary phase (Surette and Bassler, 1998). In AI-2 synthesis, adenosylmethionine is methylated by a methyltransferase to adenosylhomocysteine, which is metabolized to adenine and S-ribosylhomocysteine (Miller et al., 2004). S-ribosylhomocysteine is cleaved by LuxS to generate homocysteine and 4,5-dihydroxy-2,3-pentadione, which may spontaneously form two different forms of AI-2 (Miller et al., 2004). Adenosylmethionine is also an important cofactor for PflA in dissimilation of pyruvate to acetate (Kessler and Knappe, 1996). However, when *E. coli* was grown at 6°C, levels of PflA increased while levels of PflB decreased with increasing incubation time. This suggests that cells have a decreased demand for maintaining the Pfl dependent pathway of converting pyruvate to acetate. Instead, cells may utilize adenosylmethionine for AI-2 synthesis as levels of both glucose inhibited division protein (GidB), which is suspected to have methyltransferase activity (Romanowski et al., 2002), and LuxS increased 4-fold after 4 days and 5-fold after 8 days of incubation at 6°C. Although quorum sensing is often associated with high cell numbers and the stationary phase, in the current study production of AI-2 should not be linked to increases in cell numbers because absorbance values after 8 days of incubation at 6°C were similar to those measured for the control culture prepared at 15°C. These findings support the suggestions that AI-2 maybe involved in metabolic processes that slow growth and that AI-2 may interact with other signals such as starvation to regulate the entry into the stationary phase (Huisman and Kolter, 1994; Lazazzera, 2000; Sperandio et al., 2001).

4.4.8 Protein stability

Ribosomal and other structural proteins, and regulatory proteins require a precise conformation to carry out their function. Molecular chaperones protect cells from thermal stress by binding to the exposed hydrophobic amino acid residues of unfolded, misfolded or aggregated proteins and either assist them to refold properly or target them for destruction (Bukau and Horwich, 1998; Georgopolous and Welch, 1993). Protein misfolding is accepted as a major problem at high temperatures but it was not considered a major problem at low temperatures until it was recognized that the molecular chaperones trigger factor, Hsc66 and Hsc20 are induced in response to cold temperatures above the minimum for sustained growth (Kandror and Goldberg, 1997; Lelivelt and Kawula, 1995). When cold adapted, log phase *E. coli* were incubated at temperatures below the minimum for sustained growth, changes in levels of proteins associated with protein folding, such as peptidyl-prolyl cis-trans isomerase B (PpiB), FKBP-type peptidyl-prolyl cis-trans isomerase (SlyD), GroES, and disulfide bond isomerase (DsbC) were observed.

The catalysis of cis/trans isomeration of peptide bonds by PPIases is essential for protein synthesis and folding to continue at low temperatures, as isomeratization is often a rate limiting step in the folding of polypeptides at low temperatures (Hesterkamp and

Bukau, 1996; Stoller et al., 1995; 1996; Teter et al., 1999). Levels of PpiB were unchanged after 1 day of incubation at 6°C relative to levels expressed at 15°C but levels increased after 4 and 8 days of incubation at 6°C. In contrast, levels of SlyD decreased 5-fold after 1 day of incubation and remained at low levels with increasing incubation time at 6°C. However, the overall concentration of SlyD and PPiB together remained fairly constant in the cytoplasm, accounting for about 0.7% of the total amount of protein, which suggests that cells may substitute PpiB for SlyD at 6°C. While increased expression of PpiB has not previously been demonstrated for *E. coli* at low temperatures, PpiB levels in *Bacillus subtilis* were elevated for the first 90 min after cold shock at 15°C and subsequently returned to levels produced at 37°C (Graumann et al., 1996). In the current study, levels of DsbC increased slightly after 1 day of incubation at 6°C but were 4 times higher after 4 and 8 days of incubation at 6°C and levels of GroES increased approximately 2-fold after 1 day of incubation at 6°C and remained constant with increasing incubation time. It appears that activity of GroEL/GroES must be tightly regulated as an increased demand for activity of GroEL/GroES also increases the demand for ATP. The increased demand for enzymes associated with protein folding at 6°C suggests that cells require increased assistance with proper protein folding, which places an additional energy burden on cells.

Proteins that cannot be properly folded or refolded are degraded by proteases. Slightly higher levels of ClpP, Lon, and peptidase B (PepB) were observed after 1 day of incubation at 6°C but levels of PepB increased 7-fold after 4 days of incubation at 6°C, while levels of Lon increased 3-fold after 8 days of incubation. Moderate increases in protease levels have a dramatic influence on cells. Lon and ClpP levels increase 2- to 3-fold during induction of the heat shock regulon, and a 5-fold increase in Lon levels is inhibitory for growth (Goff and Goldberg, 1987; Kroh and Simon, 1990). Lon and Clp are ATP dependent proteases which interact with the substrate at specific substrate recognition sites to degrade the substrate to small peptides without producing potentially detrimental protein fragments (Miller, 1996). Small peptides can be further hydrolyzed to free amino acids by peptidases. Amino acids may serve as potential carbon and energy sources (McFall and Newman, 1996). However, L-serine is toxic to the cell in high concentrations and is readily degraded by L-serine deaminase (Sda; Uzan and Dancin, 1978; Prüß et al., 1994). Indeed, SdaA levels were 2-fold higher after 4 and 8 days of incubation at 6°C. Increased levels of chaperones and proteolytic enzymes with increasing incubation time at 6°C to levels similar to those observed during the heat shock response suggest that protein stability may be a significant problem at temperatures below the minimum for sustained growth, which drains a significant amount of energy from the cells. Increased levels of oligopeptide permease (OppA) with increasing incubation time suggests that cells may be able to improve growth and energy efficiency by increasing the peptide scavenging capacity (Goodell and Higgins, 1987).

4.4.9 Carbon metabolism

Pyruvate can be utilized as a source of carbon for biosynthetic pathways and as a source of energy. Pyruvate dehydrogenase and pyruvate lyase (PflB) are essential enzymes for the oxidation of pyruvate to acetyl-CoA during aerobic respiratory growth and anaerobic fermentative growth, respectively, and are not associated with the respiratory chain (Gennis and Stewart, 1996). Acetyl-CoA can subsequently be converted to acetate for biosynthetic pathways or enter the TCA cycle. In contrast, pyruvate oxidase (PoxB) utilizes pyruvate as an energy source, which appears to be wasteful, yet PoxB actually appears to contribute to the energetic efficiency as a lower specific growth rate is observed when *poxB* is inactivated (Abdel-Hamid et al., 2001). The role of PoxB is not clear but PoxB may be important for cell survival during the transition from the exponential to the stationary growth phase as *poxB* expression is very low during exponential growth and is regulated by RpoS (Chang et al., 1994). PoxB levels were very low in cold adapted, log phase *E. coli* cultures at 15°C, but levels increased 4-fold after 1 day of incubation at 6°C and remained nearly constant with increasing incubation time at 6°C, which indicates that pyruvate is being utilized as an energy source. The utilization of pyruvate as an energy source is further supported by decreasing levels of PflB with increasing incubation time at 6°C. PflB is inducible under anaerobic conditions while pyruvate formate lyase activating enzyme 1 (PflA) is produced constitutively during aerobic growth (Kessler and Knappe, 1996). However, when *E. coli* cultures are incubated at 4°C for 4 h prior to freezing levels of PflB are 4-fold higher than when *E. coli* cultures are frozen directly after incubation at 37°C

(Mihoub et al., 2003). Differences between those findings and this study may be related to the length of time of exposure to temperatures below the minimum for sustained growth.

A reduction of levels of aspartate ammonia lyase (AspA) and fumurate reductase (FrdB), which are enzymes involved in the reductive branch of the TCA cycle under anaerobic growth, with increasing incubation time at 6°C further supports the use of pyruvate as a carbon-energy source. Moreover, levels of both acetate kinase (AckA) and enzyme I of the phosphotransferase carbohydrate transport system (PtsI) decreased with increasing incubation time at 6°C. AckA reversibly catalyzes the conversion of acetylphosphate to acetate and transfers the phosphate group to PtsI (Kessler and Knappe, 1996). Crr protein is phosphorylated by PtsI and histidine protein (Hpr) and the phosphorylated form of Crr negatively controls translation of *rpoS* (Ueguchi et al., 2001). As levels of PtsI decreased during incubation at temperatures below the minimum for growth, phosphorylation of Crr would likely also decrease, which could affect the derepression of *rpoS*.

In contrast, levels of TCA cycle enzymes citrate synthetase (GltA), succinyl-CoA synthetase (SucC), 2-oxoketoglutarate (SucB) and malate dehydrogenase (Mdh) increased after 1 day of incubation at 6°C and continued to increase with increasing incubation time at 6°C. The full TCA cycle is usually operational during aerobic growth on acetate or fatty acids, providing the cell with all the energy and reducing potential required to support growth (Cronan and LaPorte, 1996). A branched TCA cycle is active in aerobic cells growing on glucose or rich media, as some TCA cycle enzymes may be subject to endproduct repression by amino acids (Cronan and LaPorte, 1996). It appears that, at 6°C, the full TCA cycle is used to provide a source of energy, as levels of SucB and SucC increased 5- and 10-fold, respectively, after 8 days of incubation at 6°C. SucC is not required for the branched biosynthetic pathway while GltA is activated by NADH and may be rate limiting enzyme of the TCA cycle when *E. coli* is grown on a poor carbon source such as acetate (Cronan and LaPorte, 1996).

In the oxidative branch of the pentose phosphate pathway, carbon compounds can be used as an energy source to generate NADH (Fraenkel, 1996). Levels of transaldolase B, which is associated with the pentose phosphate pathway, also increased slightly with after 4 days at 6°C, which suggest that at 6°C, some carbon compounds may be diverted to the pentose phosphate pathway as a source of energy. Decreased levels of glucosamine-6-phosphate synthetase, which is involved in converting fructose-6-phosphate to D-glucosamine for the biosynthesis of peptidoglycan and lipopolysaccharides, were observed after 4 days of incubation at 6°C. This indicates that cells at 6°C require less fructose-6-phosphate for biosynthesis than cells at 15°C, and that intermediates of both the glycolytic and the pentose phosphate pathway are preferentially being utilized as sources of carbon for energy generation (Lin, 1996).

Ribonucleosides formed as a result of mRNA turnover by RNases, such as RNase PH, can be utilized for nucleotide synthesis or degraded as a source of carbon (Zalkin and Nygaard, 1996). Gradual increases in levels of RNase PH and delayed increases in levels of PurB and immediate decreases in levels of IMP dehydrogenase and nucleoside diphosphate kinase suggest that in cells grown at 6°C, nucleotides are also being utilized as a source of carbon for energy generation (Zalkin and Nygaard, 1996).

4.4.10 Electron transport

E. coli adapts the composition of its respiratory system in response to different growth conditions. Electrons flow via dehydrogenases from donor substrates to a common quinone pool and are passed on to a terminal oxidoreductase, which reduces the terminal electron acceptor that is being utilized (Gennis and Steward, 1996). The amount of each component is tightly regulated to optimize the electron transport chain (ETC) according to the substrates present and the physiological needs of the cell. Thus factors such as redox state of the cell and the need to regenerate NAD^+ from NADH and bioenergetic efficiency affect the elements of the ETC (Gennis and Steward, 1996). Levels of several proteins associated with the electron transport chain, such as quinolate phosphoribosyl transferase, pyridine nucleotide transhydrogenase (SthA), hypothetical oxidoreductase (YjgI) and thioredoxin increased moderately with increasing incubation time at 6°C but levels of the hemoprotein dihydropteridine reductase (Hmp; Andrews et al., 1992; Kadner, 1996; Lim et al., 1985) increased almost 100-fold after 8 days of incubation at 6°C.

Hmp, a flavohemoprotein with NADH oxidase activity, is an unusual soluble oxidase that is synthesized at very low rates under favourable growth conditions in addition to the two membrane-bound terminal oxidases cytochrome bo' and bd, which are synthesized under high or limited oxygen concentrations, respectively (Poole et al., 1994; 1996). Hmp is proposed to act as an oxygen sensor, as the affinity of Hmp for O_2 is lower than that of cytochromes bo' and bd. Hmp may then become active when the intracellular O_2 concentration becomes rate limiting (Poole et al., 1994; 1996). In the presence of O_2, the oxidized protein reacts with NADH to form an oxygenated product while FAD remains oxidized. When O_2 becomes depleted, the oxygenated haeme converts to a deoxy form, with reduction of FAD (Poole et al., 1994). Increased expression of Hmp in *E. coli* has been linked to oxidative and nitric oxide stress (D'Autréaux et al., 2002) but has not previously been linked to low temperature stress. Although the SoxRS regulon responds to superoxide generating agents and nitric oxide, the induction of *hmp* is independent of SoxRS (Poole et al., 1996; Membrillo-Hernandez et al., 1997b). Hmp accelerates O_2 uptake when induced by NO, NO_2^- and NO_3^- and protects cytochromes bo' and bd from NO through NO oxygenase activity (Stevanin et al., 2000). RpoS may exert some regulatory control over *hmp* expression as increases in *hmp* expression levels were lower for an RpoS mutant strain compared to the wild-type strain during the stationary phase in the presence of paraquat, a superoxide generating agent, but expression levels were similar during the exponential phase of growth (Membrillo-Hernandez et al., 1997b). However, *hmp* was not induced in a *relA spoT* mutant of *E. coli* when cultures approached the stationary phase, which suggests that *hmp* is regulated by ppGpp (Membrillo-Hernandez et al., 1997a).

The expression levels of *hmp* increase approximately 30- or 40-fold in the presence of 2'2'-dipyridyl, a Fe^{2+}chelator, in the exponential or stationary phase of growth, respectively, during aerobic conditions (Poole et al., 1996). In *Salmonella* Typhimurium, *hmp* expression is induced by NO in a ferric uptake regulation (*fur)* mutant (Crawford and Goldberg, 1998). The Fur regulon is also induced in response to NO stress in *E. coli* and *Salmonella* (D'Autréaux et al., 2002; Crawford and Goldberg, 1998). The active form of Fur protein, FeFur, contains a nonhaeme ferrous iron site with oxygen and nitrogen donor ligands (Adrait et al., 1999). The Fur repressor is inactivated due to the loss of the corepressor Fe^{2+} when cellular iron becomes limiting, or due to nitrosylation of Fe^{2+} in the presence of NO (D'Autréaux et al., 2002). At temperatures below the minimum for sustained growth, it is not clear whether increased Hmp levels are related to RpoS, inactivated Fur repressor in response to low iron concentrations, the presence of NO, NO_2^- and NO_3^-, or other regulatory mechanisms. Furthermore, Fur represses *hmp* but levels of Fur actually increased with increasing incubation time at 6°C. The mechanism of *hmp* regulation appears complex and is not yet understood.

A recent study demonstrated induction of PspA when activity of the proton motive force, particularly cytochrome o and the F_1F_0 ATPase complex, is reduced (van der Laan et al., 2003). The findings of this study were consistent with findings that induction of PspA coincides with alterations in the ETC and supports the suggestion that Hmp may act as an alternative oxidase under stress conditions (Kleerebezem et al., 1996; Model et al., 1997; van der Laan et al., 2003). Levels of PspA, Hmp, and a hypothetical oxidoreducase YjgI increased 3-, 6- and 2-fold, respectively, after 1 day of incubation at 6°C and much larger increases were observed for all three proteins after 4 and 8 days of incubation. PspA is not induced upon exposure to DNA damaging agents or mild stresses but is transiently induced after exposure to extreme heat, high levels of ethanol, extreme hyperosmotic shock induced by NaCl or sucrose, and agents or conditions that interfere with energy production or the cytoplasmic membrane (Bergler et al., 1994; Brisette et al., 1990; Kobayashi et al., 1998; Weiner and Model, 1994). Induction of PspA resembles the induction of the heat shock response; however, induction of PspA is not dependent on RpoH but is dependent on RpoN, the product of σ^{54} (Brisette et al., 1990; Reitzer and Schneider, 2001). Furthermore, *psp* mutants starved for carbon at high pH were limited in their ability to produce energy and died more rapidly when cocultured with wild-type cells, which suggest that the induction of PspA may be related to the induction of processes involved in energy generation or inhibition of processes that deplete energy (Weiner and Model, 1994). PspB, a PspA homologue, was also transiently induced in *Bacillus subtilis* when cultures were shifted from 37°C to 15°C and levels are reduced when growth resumes; however in this study, increases in PspA levels were not transient and continued to increase as cells were dying off at 6°C with increasing incubation time (Graumann et al., 1996).

As the cell appears to be conserving energy during incubation at 6 °C, it is not surprising to observe that levels of FliC decreased with increasing incubation time at 6°C. Flagella are nonessential structures that require energy derived from the proton motive force from the ETC for locomotion while biosynthesis of FliC has a high metabolic cost (Macnab, 1996). These findings are consistent with a report of lower levels of FliC in *E. coli* cells that were incubated at 4°C for 3 h before freezing compared to cells that were not cold adapted prior to freezing (Mihoub et al., 2003).

4.4.11 Iron metabolism

Iron is an essential element for cells and is required, among others, for iron sulfur cluster biosynthesis, which is mediated by the iron-sulfur cluster (*isc), nif*, and *suf* gene clusters (Agar et al., 2000; Beinert, 2000; Beinert et al., 1997; Patzer and Hantke, 1999). Cells appear to have an increasing demand for iron sulfur cluster biosynthesis at 6°C as levels of IscU, IscS, and SufC increased after 4 days of incubation.

However, levels of iron must be tightly regulated because iron is also involved in the formation of hydroxyl radicals through the Fenton reaction, which can damage DNA, lipids, and proteins (Earhart, 1996). Iron uptake is regulated by Fur, which negatively regulates many genes involved in ferric iron uptake from the environment. However, Fur also controls genes involved in the TCA cycle, pathogenicity, and redox-stress resistance (McHugh et al., 2003). Moreover, Fur also control a set of genes involved with acid stress and the flagellar regulon that are independent of iron levels in *S.* Typhimurium (Campoy et al., 2002; Hall and Foster, 1996). Fur synthesis is decreased by the active FeFur complex and is stimulated by cAMP-CRP, SoxRS, or OxyR (Zheng et al., 1999; de Lorenzo et al., 1988). Levels of Fur were 2-fold higher after 4 and 8 days of incubation at 6°C, which are similar to increases reported for *E. coli* exposed to 0.2 mM hydrogen peroxide (Zheng et al., 1999). This suggests that Fur levels may be increased as a protective mechanism or as a result of exposure to reactive oxygen species. The cell division inhibitor *sulA* and other genes involved in the SOS response are upregulated in *E. coli* O157:H7 in the presence of AI-2 (Sperandio et al., 2001). Furthermore, DinG, which is involved in DNA replication and repair, is induced when *E. coli* cultures are incubated at 4°C for 4 h prior to freezing but not when *E. coli* cultures are frozen directly after incubation at 37°C (Mihoub et al., 2003). As levels of Fur and LuxS, which is associated with AI-2 production, both increased with increasing incubation time at 6°C, it could be expected that some proteins associated with the SOS response would also be differentially expressed; however, this was not observed. This lack of detection of proteins specifically associated with the SOS response could be due to limitations associated with image analysis or due to the involvement of other regulatory mechanisms.

4.4.12 DNA replication

The initiation of chromosome replication must be tightly regulated and is closely linked to cellular growth. When conditions become unfavourable for growth, it is anticipated that DNA replication ceases; however, the finding that DNA replication continues at temperatures just below the minimum for growth is consistent with that found for *E. coli* and *Salmonella* at temperatures just below the minimum for sustained growth (Fedio, 1986; Mattick et al., 2003; Phillips et al., 1998; Shaw, 1968). In the studies, filamentation was attributed to an early block in the cell division genes involved in septation as regularly spaced nucleoids are present. Exposure to any environmental condition that interferes with DNA synthesis, either directly or indirectly, has the potential to induce the SOS response, which can result in a delay in cell division (chapter 1). While levels of single stranded binding protein (Ssb) increased nearly 2-fold after 4 days of incubation at 6°C, differential expression of other proteins known to be involved in DNA replication or repair was not observed. Ssb is associated with the replication fork, particularly the lagging strand, and protects the exposed single stranded DNA (ssDNA) from nuclease attack and stimulates the rate and processivity of DNA polymerase III (Marians, 1996). Furthermore, Ssb is also involved in DNA repair (Marians, 1996). Ssb has a high affinity for ssDNA and is able to lower the melting temperature of dsDNA (Marians, 1996).

Initiation of replication is negatively controlled by SeqA, which sequesters the chromosome origin to the outermembrane after replication is initiated until the new origins of replication (*oriC*) are methylated at specific GATC sequences by deoxyadenosine methylase (Dam). Initiation of chromosome replication is more efficient on fully methylated *oriC* DNA but methylation is not essential (Marinus, 1996). The *dnaA* gene is in close proximity to *oriC*, and GATC sites within both of the regions are in a hemimethylated state for about 30 to 40% of the cell cycle (Campbell and Kleckner, 1990). *E.coli* contain around a 100 molecules of Dam per cell and its levels must be tightly controlled to coordinate DNA replication with cellular growth; however, little is known about its role and regulation (Marinus, 1996). Transcription of *dam* can proceed from several promoters, promoter P2 is regulated by growth rate but is not affected by the stringent response, ribosomal feedback or the level of Fis protein (Rasmussen et al., 1995; Marinus, 1996).

While differential expression of Dam was not detected; levels of GidB, which was produced in very low quantities at 15°C, increased 5-fold after 8 days of incubation at 6°C. A physiogical role has not been assigned to GidB. This appears to be the first report of increased expression of GidB in response to stress. It would be interesting to know if GidB is also induced during exposure to other stresses. GidB could be an alternate or additional methyltransferase used to methylate DNA during low temperature stress. X-ray structure analysis of GidB indicates the presence of a methyltransferase fold and hydrophobic domains, which suggests that it may be associated with the cell membrane, (Romanowski et al., 2002). GidB is suspected to have S-adenosylmethionine methyltransferase activity, similar to Dam (Marinus, 1996; Romanowski et al., 2002).

The dicistronic *gidAB* operon is located immediately left of the chromosomal region containing *oriC* (Walker et al., 1984). GidB is considered to belong to the minimal gene set for cellular life while Dam, Dcm and HsdSM DNA methylases are not essential as viability was maintained in a *dam dcm hsdS* mutant (Marinus, 1996; Romanowski et al., 2002). Mutations in *gidA* and *gidB* results in inhibition of cell division while disruption of *gidA* decreases the growth rate in glucose media (Messer and Weigel, 1996). Although very little is known about *gidB*, it has been suggested that transcription from genes flanking *oriC* may activate the initiation of chromosome replication (Theisen et al., 1993; Zhou et al., 1997), which would explain continued DNA replication observed at 6°C. Furthermore, transcription from the *gid* promoter oscillates in the same manner as transcription of *dnaA*, which decreases after the initiation of DNA replication (Theisen et al., 1993; Zhou et al., 1997). Transcription of *dnaA* is negatively affected by (p)ppGpp at 37°C but is positively affected by low temperatures (Atlung and Hansen, 1999; Chiaramello and Zyskind, 1990).

Transcription from the *dnaA* promoter in the fully methylated state is 10 times more efficient than from the hemimethylated state (Marinus, 1996). A 2-fold higher concentration of DnaA per *oriC* is found in cells growing at 14°C than at 37°C; however, DnaA produced at 14°C has a lower activity than the DnaA produced at 37°C, but the reasons are not yet known (Atlung and Hansen, 1999). A higher concentration of DnaA protein appears to bind at the open complex formations when conditions are unfavourable for active complex formation (Langer et al., 1996; Messer et al., 2001).

Dam is also associated with postreplication mismatch repair, which involves the removal of replication errors in the new synthesized undermethylated DNA strand (Modrich, 1991). When excess Dam is present, DNA is in a hemimethylated state for a shorter period of time, which prevents mismatch repair and consequently avoids the induction of the SOS response (Herman and Modrich, 1981; Marinus, 1996). Increased production of GidB could possibly provide a protective role by preventing the induction of the SOS response to conserve the cell's resources and delay repair until conditions become more favourable for growth. Other than Lon protease, which is also upregulated during the stringent and heat shock response, differential expression was not observed for proteins specifically associated with the SOS response. If GidB acts in a protective role by preventing the induction of the SOS response until conditions become favourable for growth, then the SOS response could be induced when cells are incubated at temperatures above the minimum for growth. Induction of the SOS response during incubation at growth permissive temperatures after incubation at temperatures below the minimum for sustained growth could account for the observed increasing elongation at growth permissive temperatures, before cells divide into cells of normal size, which could be due to cell division being delayed until DNA damage is repaired. Such a delayed induction of the SOS response would also explain the lack of differential expression of proteins specifically associated with the SOS response at temperatures below the minimum for sustained growth.

4.4.13 Conclusions

The behaviour of cold adapted, log phase *E.coli* at temperatures below the minimum for sustained growth appears to be complex and involves regulators of various pathways. It appears that cells are preparing to enter the stationary phase after 1 day of incubation at 6°C. However, cells that are able to continue to grow appear to require additional assistance with protein folding and have increased energy demands, which may lead to an induction of the stringent response. However, DNA replication is not inhibited and possibly may be assisted by enhanced production of GidB. In contrast, cell division apparently is impaired. The *bolA* gene, which is regulated by RpoS, appears to be involved in the switching between cell elongation and septation systems, causing a round morphology when cells are exposed to environmental stress, which is dependent on active FtsZ (Aldea et al., 1988; 1989). The assembly of the FtsZ ring is dependent on GTP while disassembly of the FtsZ filaments occurs when GTP is depleted (Bramhill and Thompson, 1994; Yu and Margolin, 1997). Considering the fact that cells contain between 10,000 to 20,000 molecules of FtsZ, there presumably is a great demand for GTP (Erickson et al., 1996; Rothfield and Justice, 1997). During the stringent response, cells consume ATP to convert GTP into pppGpp, which could deplete cells of GTP required for cell division. Furthermore, lower levels of Ndk at 6°C decreases the cell's ability to regenerate GTP from GDP. Therefore, it is plausible that cell division is simply inhibited due to decreased levels of GTP, or perhaps due to some other indirect effects of (p)ppGpp. Although it has been proposed that the alarmone (p)ppGpp possibly helps the cell with proper timing of *rpoS* expression for normal stationary phase development (Cashel et al., 1996), that may not be the case when conditions are just at the boundary of growth.

4.5 References

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5. General Discussion and Conclusions

The temperature experienced by chilled foods is a major factor affecting their microbiological safety. While the effect of cold temperatures on the viability of microorganisms that are subjected to sharp drops in temperature or cold shock has been well documented, there is a lack of information on the effect of chiller temperatures on cells that are cooled relatively slowly. Temperatures of chilled foods may fluctuate above and below the minimum growth temperature of *Escherichia coli* and other related mesophilic pathogens. *E. coli* can grow exponentially on raw meat when temperatures remain or rise above 7°C during carcass cooling, fabrication of cuts, and display. However, the current assumption is that *E. coli* does not grow below 7°C (Shaw et al., 1971). Models for predicting the growth of bacteria in foods have been developed by monitoring changes in the absorbances of or the numbers of colony forming units (cfu) recoverable from liquid cultures (Adams et al., 1991; Buchanan and Phillips, 1990; Gibson et al., 1988). When models that relate the duration of the lag phase and growth rate of bacteria to the physical and chemical conditions of growth media are constructed from such data, it is necessarily assumed that the population of cells in each culture is homogeneous (Baranyi et al., 1993). While that may often be the case, the assumption is not always accurate (McKellar, 1997).

The overall objective of this research was to gain an increased understanding of the behaviour of cold adapted, log phase *E. coli* that are exposed to temperatures near the minimum for growth. This knowledge is essential for predicting microbial growth and for identification of effective methods for controlling their growth in chilled foods. The first objective was to examine the behaviour of *E. coli* at temperatures below and near to but above the minimum for growth to resolve the discrepancies between counts of cfu and absorbance data for the same culture, and to better characterize the lag phase that develops in log phase *E. coli* exposed to chiller temperatures.

The findings of these studies have shown that cooled cultures become heterogeneous with time, as some but not all cells elongated into filaments at temperatures below and near to but above the minimum for growth. Numbers of cfu decreased while absorbance values increased for cultures incubated at temperatures ≤

7°C, but at temperatures > 7°C the numbers of cfu and absorbance values increased with time. Even though growth was sustained at 8°C, the growth rate estimated from absorbance values was more than double the rate estimated from the numbers of cfu; and when cultures that were incubated at 6°C were subsequently incubated at 12°C, elongated cells increased in length before they divided into cells of normal size. The division of elongated cells to cells of normal size resulted in the numbers of cfu increasing at rates greater than the exponential growth rate at 12°C. Moreover, elongation of cells of normal length was observed in cultures returned to 12°C when A_{600} values increased during the first day of incubation at temperatures below the minimum for growth but normal growth was observed at 12°C when A_{600} values did not increase during the first day of incubation at temperatures below the minimum for growth. Furthermore, it was assumed that cultures in the logarithmic phase of growth at 12°C for > 24 h would be homogeneous in cell length because cells are reported to adapt to low temperatures, such as 10°C, within 4 h (Jones et al., 1987). However, the cold adaptation process is apparently more complex than has been understood because a subpopulation of the bacterial cells became elongated after prolonged periods of time at temperatures as high as 12°C.

The elongation of *E. coli* cells at temperatures a few degrees below the minimum for growth raises the possibility that division of filaments may occur or filamentation may be enhanced when temperatures rise regularly if, perhaps, briefly from below to above the minimum growth temperature, as commonly occurs during defrosting cycles of retail display cases for chilled foods (James, 1996). Therefore, the behaviour of *E. coli* cultures incubated at temperatures below the minimum for growth with periodic fluctuations to temperatures above the minimum for growth was examined. The findings of these studies showed that when cells were incubated at 6°C with fluctuations at intervals of $\leq$ 12 h, growth, with elongation of many cells and subsequent division of filaments, was sustained. In contrast, when cells were incubated at 4°C, loss of viability was accelerated by temperature fluctuations at 6 h intervals. When cells were incubated at 2°C, loss of viability was retarded by temperature fluctuations at 6 h intervals. Some fractions of cells elongated during incubation at 2 and 4°C, and some elongated cells subsequently divided at 4°C when temperatures fluctuated at 6 h intervals, but not when temperatures were constant. The finding that cells behave differently under fluctuating than at constant temperatures may significantly affect the understanding of appropriate temperatures for the safe storage of chilled foods and for predictive modelling of bacterial growth in such foods. The maintenance of growth by brief upward fluctuations of temperature at temperatures below that generally accepted as the minimum for growth would in some circumstances invalidate the predictions of "fail safe" microbiological models and lead to some underestimation of microbiological risks associated with chilled foods. Underestimation of the risks would arise because each elongated cell would be detected as a single colony with consequent underestimation of the potential numbers of bacteria to which consumers would be exposed when elongated cells rapidly divide at room or body temperatures (Little and Knøchel, 1994). Furthermore, the division of filamentous cells at growth permissive conditions may contribute to large differences in viable counts that can be obtained for the same food sample, depending on the time of sampling. Such large differences in microbial counts could have significant implications for the food industry as food products cannot exceed specified microbial tolerance levels set by regulatory agencies or certain customers. For example, when *Salmonella* was incubated at 8°C, filaments up to 150 μm long were formed (Mattick et al., 2003). When the culture was warmed to 37°C, a 200-fold increase in cell counts was observed without an accompanying increase in biomass (Mattick et al., 2003). Certainly, the formation of filaments by cells under such conditions may lead to risks being underestimated when risk estimates are based on the numbers of cfu recovered from foods.

Although an increase in bacterial numbers from the division of elongated cells may be of little significance with organisms that have high infective doses, even small increases in the numbers of pathogens with a very low infectious dose, such as *E. coli* O157:H7, may substantially increase risks to consumers' health. Because the behaviour of pathogens at temperatures near their minimum for growth can be complex, current assessments of microbiological risks associated with chilled foods might well be erroneous, irrespective of whether they are based on the predictions of "fail safe" models (Little and Knøchel, 1994) or direct determination of increases in the numbers of colony forming units in foods. The temperature of 4°C mandated for the storage or display chilled foods (USDA, 1996) may be inappropriate because a substantial fraction of cells elongated when incubated at 4°C with temperature fluctuations that are commonly encountered by chilled meat on retail display (Gill et al., 1998). The findings also increase concern about the large fraction of displayed chilled foods that experience persistent temperatures above 4°C. A recent study of beef in commercial retail display cases in Canada indicated that about 35% of product was displayed at temperatures above 4°C, while almost 10% of product was displayed at temperatures above 6°C (Gill et al., 2002). While the extent of filament formation of *E. coli* has not yet been determined in foods, filament formation at low temperatures of various *Salmonella enterica* serovars, including *S. enterica* serovar Enteritidis PT4 and *S. enterica* serovar Typhimurium DT104 has been observed in chicken meat extract, chicken meat surfaces, and skim milk (Fedio, 1986; Mattick et al., 2003).

The linear relationship between cell numbers and absorbance is invalid when cells experience conditions of unsustained growth or death. A better understanding of the physiological, biochemical and molecular mechanisms involved in the responses of bacteria to cold temperatures is essential for predicting microbial growth and for identification of effective methods for controlling their growth in chilled foods. The formation of filaments by some log phase *E. coli* at temperatures near but above their minimum for growth would seem to support the suggestion that changes in the physiological state of individual cells could be responsible for difficulties encountered in modelling bacterial growth when conditions are marginal (Baranyi et al., 1995). Difficulties are also encountered in the modelling of lag phase duration and bacterial growth when factors such as temperature, pH and a_w approach the limits for growth (Buchanan and Phillips, 1990; Gibson et al., 1988). The lack of fit of bacterial growth data to existing models could be partially due to the formation of elongated cells under conditions of low a_w and starvation and at low temperatures. Yet, cell elongation is generally not considered to be a factor that contributes to the difficulties in modelling the behaviour of bacteria near their limits for growth. However, the current findings have shown that cell elongation can occur over a wide temperature range and must be taken into consideration when observed growth in foods is less than that expected by predictive models. The concept of predictive microbiology requires a detailed knowledge of microbial responses to environmental conditions for realistic assessments of the effect of processing and storage conditions on the microbiological safety of foods. However, it is recognized that as a microbial population moves progressively towards conditions that will eventually limit growth, it becomes increasingly difficult to accurately predict growth (McMeekin et al., 2002).

There is a lack of knowledge of the mechanism involved in filamentation at temperatures near the minimum for growth. A single report of filament formation in *E. coli* cells at temperatures around the minimum for growth was published nearly 40 years ago (Shaw, 1968) while published reports of filament formation in *Salmonella* cells at temperatures just below the minimum for sustained growth have recently emerged (Mattick et al., 2003; Phillips et al., 1998) and was previously noted by Fedio (1986). In addition, filamentation has also been reported for gram positive and gram negative bacteria during exposure to high temperature (Rowan and Anderson, 1998), high concentrations of carbon dioxide (McMahon et al., 1998), low and high pH (Everis and Betts, 2001; Isom et al., 1995; Lemay et al., 2000), low water activity (Isom et al., 1995; Jørgensen et al., 1995; Mattick et al., 2000), hydrogen peroxide (Brandi et al., 1989; Isom et al., 1995) and starvation (Giard et al., 2000; Wainwright et al., 1999).

Filamentous growth in response to low temperature or low a_w has been attributed to a block in cell division process because regularly spaced nucleoids are present but indentations in the cell wall are not apparent (Mattick et al., 2000; 2003). However, a direct or indirect link to the mechanisms involved in the inhibition of cell division in response to various environmental stresses has not been made. A review of the literature suggests that the block in cell division at low temperatures is most likely related, either directly or indirectly, to the lack of a stable or active FtsZ ring, which can be positively or negatively affected by molecular chaperones, compatible solutes, DNA supercoiling, DNA replication and repair, and DNA segregation, and the cell division process, all of which are controlled by complex regulatory networks (Draper et al., 1998; Dri and Moreau, 1993; Guthrie and Wickner, 1990; Kandror et al., 2002; Morigen et al., 2003; Singer and Lindquist, 1998; Trusca et al., 1980). However, the exact means by which cell division may be impaired at temperatures near the minimum for growth was not clear. Therefore, the proteomic response of cold adapted, log phase *E. coli* at temperatures just below the minimum for growth was examined to gain a better understanding of the physiology of cells at such temperatures. This knowledge may also provide insight into similar behaviours observed during exposure to other stresses.

The findings of the present studies show that the behaviour of cold adapted, log phase *E.coli* at temperatures below the minimum for sustained growth appears to be complex and involves regulators of various pathways. It appears that a substantial fraction of cells are preparing to enter the stationary phase after 1 day of incubation at 6°C. However, cells that are able to continue to grow seem to require additional assistance with protein folding and have increased energy demands, which may lead to induction of the stringent response. While DNA replication is not inhibited and possibly may be assisted by enhanced production of GidB, cell division apparently is impaired. The *bolA* gene, which is regulated by RpoS, appears to be involved in the switching between cell elongation and septation systems, causing a round morphology when cells are exposed to environmental stress, which is dependent on active FtsZ (Aldea et al., 1988; 1989). FtsZ has GTPase activity and the assembly of the FtsZ ring is dependent on GTP while disassembly of the FtsZ filaments occurs when GTP is depleted (Bramhill and Thompson, 1994; Yu and Margolin, 1997). As cells contain between 10,000 to 20,000 molecules of FtsZ, there presumably is a great demand for GTP (Erickson et al., 1996; Rothfield and Justice, 1997). During the stringent response, cells consume ATP to convert GTP into pppGpp, which could deplete cells of the GTP required for cell division. Furthermore, lower levels of Ndk at 6°C decreases the cell's ability to regenerate GTP from GDP. Therefore, it is plausible that cell division is simply inhibited due to decreased levels of GTP, or perhaps due to some other indirect effects of (p)ppGpp. This might be demonstrated by analysis of levels of guanine phosphates by thin layer chromatography or HPLC (Cashel, 1994; Takahashi et al., 2004).

The involvement of the stringent response in filament formation is consistent with and may explain the observation that filament formation at low temperatures in various *Salmonella enterica* serovars occurs irrespective of whether or not strains carry the *rpoS* gene, although more extensive filamentation is observed in strains with *rpoS* (Mattick et al., 2003). While it has been proposed that the alarmone (p)ppGpp possibly helps the cell with proper timing of *rpoS* expression for normal stationary phase development (Cashel et al., 1996), that may not be the case when conditions are marginal for growth.

When *E. coli*, *Salmonella* and other nondifferentiating bacteria are starved for energy or an essential nutrient, such as a carbon, they respond by inducing the starvation stress response. The response enhances expression of high affinity nutrient utilization systems, which allows for long term survival, and produces a general cross resistance to various other environmental stresses, including unfavourable temperatures, pH, osmolarity and exposure to reactive oxygen and nitrogen species. However, starved cells are not the same as stationary phase cells. Stationary phase cells are found in cultures that have stopped growing following exponential growth in rich or nonlimiting media but the factor that limits growth is not necessarily defined or singular (Lloyd and Hayes, 1995; Moat et al., 2000). In contrast, starved cells have stopped growing in response to exhaustion of one or more nutrients, so the stress that limits growth is defined (Moat et al., 2000). Stationary phase cells generally reach higher cell densities than starved cells, which may influence the overall cellular response and long term survival. Thus the proteins expressed in stationary phase cells may or may not overlap with those expressed in starved cells (Moat et al., 2000). The initial response to carbon or energy source limitation is to express or increase expression of uptake or scavenging systems to enhance utilization of any nutrients that may become available. Continual stress results in a cell that is smaller, and more resilient and metabolically efficient than the unstressed cell (Moat et al., 2000). This response to stress is mediated by the accumulation of cAMP and ppGpp and regulated by the alternate sigma factor RpoS and other global regulators (Cashel et al., 1996). Physiological changes that occur during starvation include the expression of new or higher affinity nutrient utilization systems to scavenge the environment for carbon and energy sources; the degradation of cellular RNA, proteins, and fatty acids; alteration of the lipid composition; and the condensation of chromosomal DNA, which protects it from damage (Cashel et al., 1996; Moat et al., 2000).

If cell division at temperatures near the minimum for growth is inhibited due to a depletion of GTP, then there is the possibility that filamentation may be a general stress response to environmental conditions that are just outside those for sustained growth. Growth under such limiting conditions may also induce the stringent response as cells would require additional energy to sustain growth. However, that hypothesis will need to be tested experimentally by proteomic analysis and possibly by assessments of transmembrane electrochemical potential with appropriate dyes in combination with flow cytometry or laser scanning confocal microscopy (Lloyd and Hayes, 1995).

Attempts have been made to link the energy demands of cells subjected to various stresses to the rate of cell growth and death (Knøchel and Gould, 1995; Krist et al., 1998; McMeekin et al., 2000; 2002; Shadbolt et al., 2001). Conclusions drawn from a study which focused on biomass accumulation during environmental stress and exhaustion of nutrient sources suggested that although temperature and a_w constraints had an effect on growth rates, the constraints likely did not impose large energetic burdens because energy sources were not depleted when maximum increases in absorbance values had been reached (Krist et al., 1998). When cells were subjected to conditions of low pH and low a_w, it was concluded that cells became metabolically less active when conditions became inhibitory for growth, although a mechanism for the decreased metabolic activity was not suggested. Increased demands for cellular energy in a rich food environment has thus far not been linked to the induction of the stringent response, most likely since cells are not perceived to be "starving" under such conditions (McMeekin, 2000). However, the stringent response can be invoked when cellular demand for energy is larger than that which is generated, irrespective of the abundance of nutrients (Cashel et al., 1996).

A physiological role for GidB is yet to be determined. To my knowledge, this is the first report of increased expression of GidB in response to stress. It will be interesting to know if GidB is also induced during other stresses. GidB could be an alternate or additional methyltransferase used to methylate DNA during low temperature stress. If GidB acts in a protective role by preventing the induction of the SOS response, and so conserving the cell's resources and delaying repair until conditions become more favourable for growth, then the SOS response may be induced when cells are incubated at temperatures above the minimum for growth. Induction of the SOS response during incubation at growth permissive temperatures after incubation at temperatures below the minimum for sustained growth could account for the observed increasing elongation at growth permissive temperatures, which would be due to cell division being delayed until DNA damage is repaired. Such a delayed induction of the SOS response would also explain the lack of differential expression of proteins specifically associated with the SOS response at temperatures below the minimum for sustained growth. Alternatively, cells may need additional time and resources to rebalance intermediates of metabolic pathways to resume sustained growth. A proteomic analysis of the behaviour of cells during incubation at growth permissive temperatures after being incubated at temperatures below the minimum for sustained growth should provide insight into the requirements of the cells to resume sustained growth.

Overall, the objective of this research, to gain an increased understanding of the behaviour of cold adapted, log phase *E. coli* that are exposed to temperatures near the minimum for growth, has been met. This research has increased the awareness of the extent of filament formation over a considerable range of temperatures near the minimum for growth. This knowledge will contribute to the ability to assess the risks that may arise from the growth of mesophilic pathogens during commercial processes for the storage and display of food and provides insight into the physiological response of *E. coli* at temperatures near the minimum for growth. In addition, these findings provide insight into the difficulties that are encountered in attempts to predict bacterial growth near the limits for growth.

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